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TABLE OF CONTENTS

		PAGE
Potato Blackleg, with Special Reference to the Etiological Agent	Harry M. Jennison	1–72
Studies of South American Senecios—I	J. M. Greenman	73–110
Pod and Stem Blight of Soybean	Samuel G. Lehman	111–178
Higher Fungi of the Hawaiian Islands	Edward A. Burt	179–189
Indications Respecting the Nature of the Infective Particles in the Mosaic Disease of Tobacco	B. M. Duggar and Joanne K. Armstrong	191–212
Citric Acid as a Source of Carbon for Certain Citrus Fruit-Destroying Fungi	Arthur F. Camp	213–298
Studies in the Physiology of the Fungi. XVI. Some Aspects of Nitrogen Metabolism in Fungi	Leo Joseph Klotz	299–368
An Histological Study of Regenerative Phenomena in Plants	Cora Mautz Beals	369–384
Notes on the Physiography of North Dakota and the Conditions in Certain of Its Waters	R. T. Young	385–392
Algae from Lakes in the Northeastern Part of North Dakota	George T. Moore and Nellie Carter	393–422
General Index to Volume X		423–427

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POTATO BLACKLEG WITH SPECIAL REFERENCE TO THE ETIOLOGICAL AGENT[1]

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Introduction

The objects of the investigations presented herewith were several, but only those outstanding should be mentioned at this point. First of all, a determined effort was made to discover the relationships, one to another, of the several "species" of bacteria recorded as being the cause of the blackleg disease of potatoes. To this end a thorough comparative study of the morphology, cultural features, and physiology of some 12 strains of the blackleg bacillus was made. The cultures employed represented strains of the organism from regions widely separated geographically. Among them were the 4 "species" originally described as being the cause of the disease in question. In the prosecution of the problems arising the writer was led into a quantitative study of carbohydrate utilization by strains of the blackleg bacillus and other microorganisms. The work done in this connection constitutes an important phase of the investigations carried out. By way of extending the usefulness of the paper, the writer presents a rather full and complete diagnosis of the disease, with a discussion of the economic aspects, also a revised description of the potato blackleg parasite.

[1]An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment for the requirements for the degree of doctor of philosophy in the Henry Shaw School of Botany of Washington University.

Historical

Frank ('97, '99) was the first to publish accurate and detailed descriptions of the potato blackleg disease. One may be assured he was discussing the potato disease which goes by the name of "blackleg" and which is so common and well known in many parts of North America. He reported the cause of the disease to be a bacterium which he briefly described and named *Micrococcus phytophthorus.* Micrococci have been frequently met with in isolation plates made from potato stems and tubers affected with the blackleg disease. Many other workers have reported similar experiences, but no one has presented adequate proof that any one of the species of *Micrococcus* found present under such circumstances is pathogenic to the potato. Until such time as a species of *Micrococcus* conforming to Frank's description shall be proved capable of infecting potatoes and causing the blackleg disease, *Micrococcus phytophthorus* Frank should be considered a *nomen nudum.*

About 20 years earlier Hallier ('78) described an infectious wet rot of the potato and he discussed at considerable length the association of certain bacteria ("Vibrionen," "Bakterien," "Micrococcus") and a fungus (*Peronospora infestans* Casp.) in the affected potatoes. One cannot determine from the text which of the organisms is of primary significance, but as a mere matter of historic interest it may be noted that this writer contended that the wet rot described by him was caused by "die Produkte der Plastiden der *Peronospora infestans* Casp." While it appears unlikely that the wet rot described by Hallier is in any way related to the rot due to the blackleg germ, it is nevertheless interesting to note the mention at this early date of a potato tuber rot in which bacteria were found present.

D'Arbois de Jubainville and Vesque ('78), nearly 45 years ago, showed that they were familiar with a potato tuber rot ("pourriture cellulaire") occurring in the soil, but the bacteria were not identified as the cause; rather, the disease was thought by them to be due to an excess of nutrients and water.

A year later Reinke and Berthold ('79), as a result of studies of potato decay due to fungi, concluded that a wet rot of potato tubers existed which was not due to fungi. Furthermore, they demonstrated the association of bacteria with the wet rot described, and they also proved the infectious nature of the rot.

Burrill ('90, '93) published brief notes on a bacterial disease of potatoes, but as nearly as can be determined, the disease which he studied was the brown rot of potato, later most thoroughly investigated by E. F. Smith.

A bacterial malady of the potato was described by Prillieux and Delacroix ('90) in France, and what they took to be the causal organism was designated *Bacillus caulivorus*. The potato disease referred to by Prillieux and Delacroix was described as one which affects the stem, beginning at the bottom and working upwards. To this extent, at any rate, it is comparable to the one under consideration. No description of the cultural characteristics of the organism was presented in their paper and no statement was made concerning the method of isolation by which the organism was obtained. A few years later Prillieux ('95) briefly mentioned certain of its characteristics, stating that it developed a green coloration in some media. Laurent ('99) stated that *B. caulivorus* was probably the common saprophyte *B. fluoresence liquefaciens* Flügge. Later, Delacroix concluded, according to Prunet, that *B. caulivorus* was most probably *B. fluorescence liquefaciens* Flügge, which he thought became parasitic under certain conditions. A more complete review of the work referred to just above will be found in Prunet's ('02) paper.

One of the early contributions to our knowledge of bacterial diseases of plants in general and of the potato in particular is that of Kramer ('91), who describes quite fully a wet rotting of potatoes. He obtained pure cultures of the supposed etiological agent which he described as a spore-bearing, rod-shaped bacterium.

The potato disease described by Tyron ('94, '99) as occurring in Queensland, Australia, is probably not the blackleg disease. Rather according to Tyron's statements, it is very similar to, if not the same as, E. F. Smith's brown rot disease (see especially Tyron, '99, "Biography," p. 62, and footnote p. 63; also Smith, '14, p. 208).

Smith's first publication on the brown rot of *Solanaceae* caused by *Pseudomonas (Bacterium) solanacearum* E. F. S. appeared in 1896. From the outset there was little or no confusion of this disease with the one under discussion. This paper by Smith is one of the first thoroughgoing and accurate descriptions of a bacterial disease of the potato. Recalling that his work was

done at an early date, and at a time when foreign bacteriologists of high standing opposed the idea that bacteria could be the primary cause of disease in plants, the contribution is all the more significant.

Wehmer ('98) published extensively the results of his investigations of potato diseases. In Part 3 he describes a bacterial rot of the tuber, but he definitely takes the attitude that the bacteria found associated with the rot were not the primary etiological agents. His attitude may be taken as representative of the belief of most German botanists and bacteriologists of that time.

There occurred in the vicinity of St. Petersburg, Russia, in 1898, a potato bacteriosis which was described by Iwanoff ('99). The causal organism was described as a short, oval-cylindrical, active rod found (in the tissues) to measure $1.5\mu \times 0.5\mu$. The disease was one which affected the leaves and stems, early symptoms being manifest in the leaves. Later, the stems were affected and showed symptoms of the disease. Brown lesions appeared externally on the stems, the pith was attacked and destroyed, and the stems wilted and died. Starch was not destroyed. Iwanoff contended that the disease he described was similar to that described by Smith ('96) as due to *B. solanacearum.* It appears to the writer that he was mistaken in the view presented, and from our limited knowledge of the situation it seems more likely that the potato disease prevalent in Russia near St. Petersburg in 1898 was the blackleg disease.

Jensen ('00) investigated a bacterial disease of the potato which he referred to as "blackleg". As a result of microscopic investigations of potato plants affected with "blackleg" he concluded that the disease was caused by bacteria ("Mikrokokken").

What Smith ('14) refers to as "The French Disease" was first described by Delacroix ('01) in a succession of short papers appearing in 1901. Delacroix first stated that the potato malady due to *B. solanacearum* E. F. Smith was prevalent in France. Later, he concluded that the disease referred to above, the one for which he suggested the name "brunissure," was caused by a bacterium new to science which he named *Bacillus solanicola.* Smith ('14), however, claimed that, "Here again, it is uncertain whether we have to do with *Bacillus phytophthorus, Bacterium solanacearum,* or some third organism. The writer obtained a

culture of *B. solanicola* from Prof. L. R. Jones, to whom it was given in Paris by Delacroix, but either it never possessed any pathogenic properties, which is quite probable, or else had lost them by cultivation." The same investigator made the following statement, after having had opportunity to examine material collected and preserved by Delacroix to illustrate the disease: "It represents a fungous disease of the potato."

Part III of van Hall's ('02) doctoral dissertation ("The stem rot or blackleggedness of potato stems caused by *Bacillus atrosepticus* nov. sp.") gives a fairly complete description of the disease under discussion, together with a diagnosis of the etiological agent sufficiently detailed and complete to enable one to classify the parasite. For reasons which appear below, the writer is of the opinion that the potato disease commonly referred to throughout the United States and many parts of Europe as "blackleg" is one and the same thing as that described by van Hall. The etiological agent in all cases is probably identical and, as far as is known, should be referred to van Hall's *Bacillus atrosepticus.*

At a slightly earlier date Appel ('02) published a short paper ("Contribution to our knowledge of the bacterial rotting of potatoes"), in which he described effects of the rot, also experiments on the pathogenicity of the disease and stated that the systematic position of the causal agent of the potato rot, as well as its practical importance, would be given in a forthcoming article. About a month later another short paper was published by the same investigator (Appel, '02a), entitled "The cause of the 'Blackleggedness' of the potato." In this article he takes exception to Frank's binomial in the following words: "Wenn nun auch der Frank'sche Name *Micrococcus phytophthorus* auf den Bacillus nicht anwendbar ist, so glaube ich doch aus praktischen Grunden den Speciesnamen beibehalten zu sollen, um so mehr als es sich herausstellte, dass eine ganze Reihe von Pflanzen in charakteristischer Weise angegriffen wird. Ich nenne daher den von mir isolirten Bacillus, welcher Schwarzbeinigkeit und Knollenfaule bei den Kartoffeln hervorruft: *Bacillus phytophthorus* Appel." Nowhere in this paper, however, is there to be found a description of the morphological and cultural characters (other than those implied by the name) of the etiological agent which he had isolated. The binomial given by Appel in this publication must therefore be regarded as a *nomen nudum.*

In 1903 Appel ('03) published a complete account of the potato blackleg disease as it occurs in Germany, together with a description of the causal organism. The same investigator (Appel, '05, '06) contributed substantial additions to our knowledge of this disease.

Butler ('03) mentions a bacterial disease which he thought was possibly the same as that described in the United States by E. F. Smith under the name of brown rot.

Jones ('05), in an account of disease resistance of potatoes, presented a description of the blackleg disease and accompanied it with observations upon its occurrence in Europe. He reported that it was found in Holland, Belgium, Germany, France, and England. The first authentic record of the occurrence of potato blackleg in the United States is that of Jones ('07) who found it in 1906 on a farm in Vermont. The seed used in planting the field came from Maine. He states also: "Vague reports have frequently come to the Station in previous years as to troubles of this class . . ." This author (Jones, '07) "passed almost directly from German fields where it was prevalent, to English fields and found the malady equally common and identical in appearance with that on the continent." This statement is of especial significance in connection with the report of Johnson ('07) who claims to have gathered substantial evidence of the existence of the potato blackleg disease in Ireland. (Compare also with the report made by Pethybridge and Murphy in 1910).

Harrison ('07) published a very complete account of a potato rot occurring in eastern Canada. The symptoms of the disease described by him were, he states, quite similar to the "Schwarzbeinigkeit" of van Hall and Appel. While he attributes the disease to a bacterial species (*Bacillus solanisaprus*) new to science, the writer has reason to believe (see below) that the causal agent described by him is identical with *Bacillus atrosepticus* van Hall. Morse ('07) found potatoes affected with blackleg in Maine in 1907. In 1909 he (Morse, '09, '11) published an account of the nature of the disease, its distribution, economic importance, and its control in Maine.

Smith ('10), however, was the first American to publish a full account of the blackleg parasite. His description is based on studies made with a subculture of Appel's species which he obtained from Aderhold in Berlin, presumably a transfer from

Appel's original culture. Smith states in this article that he regards *Bacillus solanisaprus* Harrison as closely related to, but not identical with, *B. phytophthorus* Appel.

About the same time Pethybridge and Murphy ('10) published a note on a bacterial disease of the potato plant, attributing it to a new species of bacterium for which they proposed the name "*Bacillus melanogenes.*" In 1911 these investigators published a full account of the malady referred to above, together with a complete diagnosis of the etiological agent. The potato disease described by them was probably none other than the "blackleg," and there is very little, if any, doubt in the writer's mind that their *Bacillus melanogenes* should be referred to *Bacillus atrosepticus* van Hall.

Murphy ('16) gives an account of a blackleg disease of potatoes occurring in Canada, attributing the disease to *Bacillus melanogenes* Peth. & Murphy.

From a series of observations made in 1916, Morse ('17) reports having seen potato blackleg in certain of our western states, though he found it to differ in certain respects from that familiar to him in Maine. This paper is an important contribution to the literature of the subject. Of particular significance are his comparative studies of the causal organisms. The writer is in accord with Morse's conclusion that the strains studied (including cultures "received under the names '*Bacillus atrosepticus* van Hall,' '*B. solanisaprus* Harrison,' and '*B. melanogenes* Peth. & Murphy'," as well as 3 isolated from blackleg material in Maine) are identical. However, since Morse was unable to procure a trustworthy culture of *B. phytophthorus* Appel he presents no data which "bear on the relationship between the organism originally described by Appel as *B. phytophthorus* and the other strains of blackleg bacteria." So in deference to the opinion of Smith ('10) that *B. phytophthorus* Appel is not identical with *B. solanisaprus* Har., Morse was forced to exclude it from *B. atrosepticus* van Hall. This is most regrettable, since Morse shows that small differences in the physiology of bacteria are not sufficient for the establishment of new species. His work shows abundant evidence of having been carefully done. Unfortunately, there seems to be no record of the comparative studies upon which Smith ('10) bases his opinion.

Paine ('17) referred to the cause of the potato blackleg studied by him in England as *Bacillus atrosepticus* van Hall and ex-

pressed the opinion that the species described by Appel, Harrison, and Pethybridge and Murphy should be referred to van Hall's *Bacillus atrosepticus.* The argument advanced by this investigator is most logical, but attention is called to the fact that he did not carry out comparative studies using the several "species" in question.

Rosenbaum and Ramsey ('18) contributed the results of some studies upon the influence of temperature and precipitation on the blackleg of potato.

Ramsey's ('19) studies of the viability of the potato blackleg organism led him to believe that the pathogen does not live in the tubers left in the field over winter. He expressed the further conclusion that unless the potato seed were infected at planting time there was little chance that the uninjured plants would contract the disease.

Artschwager's ('20) researches upon the pathological anatomy of potato blackleg form a new contribution to our general knowledge of this disease. He studied only blackleg plants grown in the arid regions of western Colorado. The affected plants examined by him were found to show an increase in strongly lignified vascular tissue and a transformation of part or most of the parenchyma cells of the cortex and pith into sclerids. This investigator also discovered that protein crystals occurred in great abundance in all organs of plants affected with the blackleg disease, especially in the leaves, while under normal conditions protein crystals have been observed only in the peripheral cell layers of the cortex of the potato tubers.

Jennison's ('21) abstract, "*Bacillus atrosepticus* van Hall, the cause of the blackleg disease of Irish potatoes" was based on the investigations carried out in large part in 1916 and 1917, here reported upon in detail. While the group number as published by the writer at that time differs in respect to the last 3 digits from that of Morse ('17), it agrees throughout with that assigned by Shapovalov and Edson ('21). The last-named investigators contributed one of the most important of the recent accounts of the disease under discussion. They showed that the potato blackleg disease prevailed in the irrigated districts of the West, and that the causal agent was a *Bacillus,* which they concluded was "identical with *Bacillus phytophthorus* Appel in all the essential characteristics considered in determining bacterial spe-

cies." A detailed description of the causal organism isolated by them was given.

I. Diagnosis of the Disease

As indicated in the preceding section, the blackleg disease of potatoes is a common and destructive bacterial disease of many varieties of *Solanum tuberosum.* The common name "blackleg" has been widely used in the United States and Canada, since it was first introduced by Jones in 1905. The term "blackleg" is a rather free translation of "Schwarzbeinigkeit," under which name the disease is known in Europe. The writer feels that blackstem and rot would be more suitable for American use but sees no point in suggesting that a change be made at this time, because (1) the old name has become well established and (2) because of its adoption by the American Phytopathological Society's Committee on Common Names for Plant Diseases. It may be pointed out, however, that the writer has observed that the term "blackleg" leads some farmers and others, especially in the West, to think that it may be related to the blackleg disease of cattle. More particularly is this the case when they learn that both are caused by bacteria.

Type—Potato blackleg is primarily a parenchyma necrosis, the cortical and pith tissues of both stem and tuber being almost exclusively involved. Occasionally, the vascular elements are found to be invaded by the parasite and sometimes they are somewhat browned.

Signs of blackleg in the field.—Considerable importance attaches to general manifestations of the disease as it appears in the field, since upon such manifestations depends the important practice of roguing, advocated below. Not infrequently "skips" or "missed hills" are due to this disease, for under favorable circumstances the young sprouts are destroyed by the parasite before they appear above ground; also the seed piece may be destroyed before it sprouts. When the disease progresses rapidly the plants wilt and become blackened to a considerable height. Such plants are quickly prostrated and soon die (pl. 1, fig. 3).

Secondary symptoms of the disease are likely to be the first to appear. Undersized vines with more or less yellowed and rolled leaves should be regarded with suspicion and more closely examined for the presence of black lesions upon the stem, both above

and below ground. There is, furthermore, a noticeable tendency for the upper leaves in particular to manifest a somewhat xerophytic texture and a starkness of growth-habit, the total effect being to give the tops of affected plants a narrowed or contracted appearance. Besides being light-colored, the upper leaves are often rendered more conspicuous by the presence of a metallic luster. Plants affected with blackleg, as a rule, offer little resistance to removal from the soil, and not infrequently break off when one attempts to pull them. If upon removal from the earth dark-colored cortical lesions are found on the underground portions of the stem, there is little doubt but that the plant is attacked by the blackleg organism. When upon splitting the stem longitudinally the pith is found blackened and more or less disintegrated the case is quite clear (pl. 1, figs. 2 and 4). Further confirmation is afforded if the seed piece has disintegrated. Aerial tuber development due to attack by the blackleg parasite is not commonly seen. The production of aerial tubers does take place when practically all the tuber-bearing stolons on well-developed plants are destroyed by the pathogen, thus precluding normal tuber development. *Rhizoctonia* more frequently causes such abnormalities of tuber development, and the underground lesions due to this fungus may be confused with those due to *Bacillus atrosepticus.* Certain characteristics of the *Fusarium* wilt disease may also be confused with the yellowing and wilting of potato vines caused by the blackleg parasite.

As a rule, plants affected with blackleg occur scattered promiscuously over the field. The occurrence and spread of the disease appear to bear a definite relation to environmental conditions, and the number of diseased plants is likely to be greater during a cold wet spring than in a warm dry one. Not infrequently there are more diseased plants in the low, poorly drained spots in a field.

"Vine" Symptoms.—The primary symptoms of the disease as they occur on the potato vines are most striking. These appear first, as a rule, on the lower parts of the stem both below and above ground, and are characterized by dark brown to black lesions or cankers, hence the origin of the German name "Schwarzbeinigkeit," or "blackleggedness." The first cankers are likely to develop below ground and not infrequently have their beginning at the lowermost part of the stem. Under favorable circumstances the disease works rapidly upward. On young succulent

stalks a lateral development of a lesion promptly results in a girdling of the stem, causing death. Under natural conditions the stem is seldom, if ever, first infected above ground. There is little tendency for the pathogen to migrate downward, even when stems are artificially inoculated at a point above ground. This fact is strongly emphasized by Smith's ('20) figures 193 and 199. The strong tendency on the part of the parasite to migrate upward along the main stem is shown in pl. 1, fig. 1. This photograph illustrates a rather striking, though not uncommon, case. Upon closer examination it will be found that the bacteria are largely confined to the parenchymatous tissues of the stem to the cortex and the pith. As it works upward in the pith this colorless parenchyma also becomes blackened and necrotic (pl. 1, fig. 4), easily observed by splitting the stem longitudinally. In this manifestation we have one of the most striking as well as reliable diagnostic features. The pith finally disintegrates, leaving the stalk more or less hollow. Frequently worms will be found feeding among the dead pith cells.

Lateral spread in large mature stems is very slight. Even though the stalk is severely diseased the plant may persist and mature a crop of tubers (pl. 2, fig. 3). Under favorable circumstances the infective agent moves through the stolons to invade the tubers. Sometimes the latter organs are infected at other points, invasion probably taking place for the most part through the lenticels, as has been experimentally demonstrated by Smith ('20). Diseased tubers frequently rot in the ground, the entrance of saprophytes bringing about the profound, slimy, putrid, soft-rot frequently observed. Death of the roots is secondary and follows a killing of the tops.

Tuber symptoms.—As implied above, stem-end infection of the tubers is most frequent under ordinary circumstances. As a rule, decay begins at the point of attachment of the stolon (pl. 2, figs. 3 and 4). Often, however, comparatively little decay is visible. Experiments carried out by the writer showed that the pathogen may be securely lodged in the tuber at the juncture with the rhizome, even though there are no signs of rot. He has also determined on many occasions that the blackleg parasite is the cause of more or less discoloration in the tissues associated with the vascular elements in the stem-end of the tubers. Such signs of the disease may readily be mistaken for symptoms of the *Fusarium* and *Verticillium* wilt diseases. In-

fection of the eye or bud end is less commonly found. A stem-end rotting of the Russet Burbank (Netted Gem) variety, due to *Bacillus atrosepticus,* is well known in many of the irrigated sections of the Northwest. It is most frequently seen at digging time, though it may be found earlier, especially if the latter part of the growing season is cool and rainy. Affected tubers are often found having pointed ends, a characteristic of some value, even though not proof-positive, since signs of the rot are more or less masked in freshly dug potatoes. Shrinkage of the affected tissues follows exposure of the tubers to drier surroundings and a brown to black discoloration shows through the skin. The presence of the rot is often exposed through breaking of the skin in the process of digging. Upon closer examination of affected tubers it will be found that the infection spreads more or less irregularly. In freshly dug tubers the rot may be described as a "soft rot" and is accompanied by a more or less putrid odor, but the blackleg as such is not to be confounded with this phase. Mixed infections, including the blackleg organism along with saprophytes and probably *Bacillus carotovorus,* are responsible for such conditions.

Dissection of tubers affected by the blackleg rot reveals the fact that the tissue may be involved to a considerable depth, extensive lesions often reaching the center. The color of necrosed tissues ranges from nearly normal to brown and black, but upon exposure to air these turn dark brown to black very rapidly (pl. 2, figs. 2 and 4). Advance of the rot is usually most rapid just beneath the epidermis (pl. 2, fig. 2), but in general spread in the tubers is not confined to any single region or tissue. Infected round tubers are often found to have a dark-colored hollow center.

A soft, cheesy rot caused by the blackleg bacillus is often seen among freshly dug tubers, though by some this type of rot is supposed to be due to "sunscald." The rot of tougher consistency found in affected tubers in transit or in storage is sometimes confounded with rots caused by *Fusarium* spp. Some very excellent illustrations of the tuber rot, including one colored plate, are those published by Shapovalov and Edson ('21).

Laboratory diagnosis.—To confirm the field observations it was necessary to make experiments in order to isolate the causal organism and prove its specific identity. Since sapro-

phytes quickly follow *Bacillus atrosepticus* in the tissues of both stem and tuber, it is often difficult to obtain the pathogen. By using extra precautions in carrying out the ordinary isolation methods one may obtain pure cultures of the causal organisms. A positive identification of the organism may then be made by careful comparison with the description of *B. atrosepticus* on p. 43.

ECONOMIC ASPECTS

Smith ('20) has come to regard the disease under consideration "as one of the most serious diseases of the potato." Earlier writers on the subject have unanimously emphasized its economic importance. Many cases are reported in the literature where upwards of 50 per cent of the plants in a field were destroyed by this disease. However, it appears that a loss of 5 per cent of the plants in a potato-growing district represents the usual amount of damage done by blackleg. In districts where the disease has been long known and where control measures have been intelligently applied, the losses have been greatly reduced, the average for the United States as a whole being about 0.5 per cent annually.

Dispersal and infection.—Probably the blackleg pathogen is most usually disseminated by the more or less general use of infected seed stocks. Morse ('16) mentions a striking illustration of this in a field in Idaho where he found blackleg which "undoubtedly came all the way from Scotland . . . in five years."

Without doubt the etiological agent is carried by wind and water, especially the latter, in the irrigated fields of the West. As pointed out by Smith ('20), invasion of the tubers through water-gorged lenticels can take place, but whether it does or not under natural conditions in the field is not known. The writer has planted healthy seed pieces in heavily infected soil but the disease did not develop in the plants thus grown. Similar results were recently attained by Shapovalov and Edson ('21). It appears to the writer that it still remains to be proven that the blackleg parasite can gain entrance to the vines or tubers through an unbroken epidermis.

The larvae of insects have been found by the writer working in and on the affected tissues, but there was no positive evidence that they wele a tive agents in the dispersal of the disease. Von

Hegyi ('10) studied the disease on the continent of Europe, and since he found that every case of "blacklegged" stalks examined bore evidence of the attack of wireworms, he thinks insects are a positive factor in the invasion of the host. Paine ('17) believes that wireworms and biting insects are undoubtedly instrumental, under certain conditions, in introducing the parasite from the soil.

Geographical distribution.—The potato blackleg disease is now known to be widely distributed in the United States, Canada, and Europe. It was reported first in the northeastern United States and eastern Canada. Its spread, however, has been rapid, especially throughout the northern states. The disease has been reported from nearly every state in the Union. The records show, however, that it is essentially a cool-climate disease, therefore prevailing in more southerly districts only where potatoes are grown at high altitudes.

The writer finds that this disease in Montana and other sections of the Northwest is caused by the same organism known to prevail in the northeastern and North Central States. The recent work by Shapovalov and Edson ('21) proves that the same organism is widely distributed in the West.

Control.—Control measures should be inaugurated during the growing season. The writer strongly advocates the planting of a portion of the crop in a separate, if possible isolated, plot. This practice enables the grower to detect, remove, and destroy diseased hills at an early date.

Inspection and roguing of the plot (or field) should be begun soon after the plants are up and repeated at more or less frequent intervals throughout the growing season, especially during cool, rainy weather. Intelligent roguing will eliminate all hills where vines show any symptoms of the disease. Final inspections should be made just before and after digging. No tubers showing signs of rot or mechanical injury should be placed in storage. It is highly important that all seed stock be stored under favorable conditions. Slatted storage bins 8 or 9 feet high, 5 or 6 feet wide, and of any convenient length are ideal. In order to permit proper aeration of the potatoes, such bins should have false floors and must be separated from each other as well as from the walls of the cellar. The cellar should be fairly moist but provision should be made by proper regulation of ven-

tilators to prevent the atmosphere from becoming saturated. As nearly as practicable the temperature should be kept at about 38° F. in order to check sprouting. Butler ('19) has shown that potatoes kept at 40° F. will sprout after about 200 days, while at 35° F. sprouting is delayed indefinitely. Shapovalov and Edson ('19) showed that potato tubers which are in a wilted or softened condition when cut, due to the development of sprouts or improper storage, are very much more likely to be infected by fungous or bacterial parasites which exist in or may be introduced into the soil.

The seed pieces to be used for planting should be sorted and only sound, rot-free tubers used. Previous to cutting they should be treated in a 0.1 per cent corrosive sublimate ($HgCl_2$) solution or in a 1:240 formaldehyde dip. If the former is used, the tubers should be immersed for 1½ hours, then more of the dissolved mercuric chloride added at the rate of 1.5–2.0 grams (depending upon the amount of dirt accompanying the tubers) for each bushel treated. Avoid treating excessively dirty potatoes in the corrosive sublimate, and make up a fresh solution after having treated 50 bushels. If the formaldehyde solution is used the tubers should be immersed in the dip for about 1½ hours. Undoubtedly, a less lengthy immersion would be sufficient to kill all contaminating blackleg germs present. When the pathogen is lodged internally treatments sufficient to kill it would probably kill the buds also.

When and where practicable, planting should be done late enough to avoid having the crop sprout and struggle along in a cold, damp soil, since it has been shown that these conditions facilitate the development of the blackleg parasite. While it has not been conclusively proved that the blackleg parasite does not overwinter in the soil at times, it is nevertheless advisable to practice rotation of the crop, since some of the worst enemies of the potato accumulate in the soil and persist therein for long periods of time.

Tests made by the writer show that the blackleg bacillus is resistant to considerable extremes of cold. Test-tubes containing about 15 gms. of soil were thoroughly autoclaved and the sterility of the soil carefully tested before inoculation. Finally, a series of cultures thus prepared were inoculated with a freshly invigorated strain of the organism and placed out of doors for 24

hours. During this time the official minimum was —28° C. and the maximum —6.7° C. Soil cultures similarly inoculated were incubated at 28° C. The cultures exposed out of doors were placed on ice upon being brought in, in order to prevent excessively rapid thawing. Finally transfers were made to nutrient broth, and agar slants from all the cultures included in the experiment. The organism was recovered from all the cultures. The subcultures made from soil which had been frozen for 24 hours appeared to be as vigorous as any.

There are few satisfactory data on varietal resistance of potatoes to the blackleg pathogen. An early contribution to this phase of the problem was made by Appel ('03), who concluded that the thick-skinned, late varieties which were being grown in Germany were more resistant to the disease than the thin-skinned, starch-poor, early varieties. Morse ('17) reported that the Irish Cobbler and Green Mountain varieties were particularly susceptible to the disease. The Early Ohio, an early variety, is notably susceptible. The Russet Burbank and the Idaho Rural, varieties widely grown in the West, are commonly found affected with blackleg.

THE ETIOLOGICAL AGENT

The writer's studies on the etiology of the potato blackleg disease were begun in Montana some years ago. By the close of the summer of 1915 extended observation led to the assumption that the blackleg disease in Montana was quite similar to that occurring in some of the eastern states. In the meantime some 30 isolations were made at the Montana Experiment Station from potatoes affected with blackleg. The pathogenicity of the strains thus isolated was thoroughly tested for the second time in 1916, and a majority was found to be pathogenic and capable of causing typical symptoms of the disease. Comparative studies of the morphological, cultural, and physiological features of some of the above-mentioned strains were made by the writer. The results obtained led to the conclusion that all were essentially alike. An extension of these preliminary investigations was made, and cultures of the blackleg bacillus from Maine, Minnesota, and one from eastern Canada received under the name *B. solanisaprus* Harrison, were cultivated and compared with one another and with observations made previously on the Montana strains. While the observations made at this time were

not extended, the writer was led to assume that a single organism caused the potato blackleg disease in North America.

A study of the literature was begun, and it was soon learned that at least 4 different species of *Bacillus* had been described by as many authors as the cause of the disease under consideration.

By comparing the following descriptions it will be seen that the "species" in question do not differ markedly. In the summaries presented no attempt is made to follow the original form, but accuracy of statement is preserved.

Bacillus atrosepticus van Hall.—The following was prepared from a translation[1] of van Hall's ('02) original description. It bears comparison with that prepared for Morse ('17) by Dr. R. de Zeeuw.

Morphology: *B. atrosepticus* is a rod-shaped bacillus, occurring for the most part singly, rarely in pairs, in 2-day-old bouillon cultures at 27°C. Size variable; length 0.8–1.6 μ, breadth 0.2–.4 μ. Many zoogloea of 4–10 organisms. The bacteria are very active in a 24-hour culture of distilled water plus 0.025 per cent potassium phosphate and 0.25 per cent asparagine (27°C.). Material stained by Loeffler's method. Length of flagella 10–15 μ. Gram negative.

Cultural characteristics: Gelatin liquefied, rapidity variable. Growth on malt gelatin and malt agar very weak. Milk coagulated. Growth best at top of meat agar stab.

Physiology: Growth very strong at 27°C. Thermal death point 51–52°C. Facultative anaerobe. Reduction of methylene blue weak. Nitrates reduced to nitrites. Sodium selenite reduced rapidly. No diastatic action. No indol production. No H_2S produced in broth cultures. The organism is a weak gas producer (except when mannite is present). Gas produced from glucose, saccharose, and mannite in a medium made by adding to "due water" (a filtered water) 0.025 per cent K_2HPO_4, 1 per cent peptone and 3 per cent of sugar. No gas from lactose and glycerin in same medium. Growth slow at first in bouillon acidified to a reaction of 0.5 per cent normal with citric and malic acid. Organism grows poorly when transferred from dried cultures: thought not to be resistant to drying. Pathogenic to potato stems and tubers. Index No. 5312-32120-2121.[2]

Bacillus phytophthorus Appel.—The following is summarized from Appel's ('03) diagnosis:

[1]My thanks are due to Dr. J. C. Th. Uphof for assistance rendered in translating Part III of van Hall's ('02) dissertation.

[2]"Index Number" digits throughout this paper are arranged according to the Descriptive Chart indorsed by the Society of American Bacteriologists, Dec. 30, 1920.

Morphology: *Bacillus phytophthorus* a fairly thick, colorless, rod-shaped organism. Breadth averages 0.8 μ, length 1.2–1.5 μ. Usually occurring singly, occasionally in pairs. Motile by means of (usually six) peritrichic flagella, stained by Peppler method. Gram negative.

Cultural features: Liquefaction of gelatin saccate. Gelatin colonies small, yellow-white, with entire margin. Agar colonies small, glistening, bluish-opalescent. Growth on agar stroke rapid, moderate to abundant. Upon sterilized potato plugs a weak honey-yellow growth develops. On sterile raw potato slices, growth and browning in 15–18 hours after inoculation. In 2 days middle line sinks and surrounding tissue becomes dark on upper surface. A liquid is pressed out. Growth in potato juice rapid and abundant. Growth in nutrient broth moderate, turbid and sediment.

Physiology: Gas from sucrose and maltose. No gas in shake cultures of sugar agar. Nitrates reduced to nitrites. Milk coagulated slowly, acid (?) curd. Pathogenic to potato stems and tubers. Index No. 5?12-32120-???1.

Smith's description of B. phytophthorus Appel.—Inasmuch as Appel's ('03) original diagnosis of his species is meager, the following compilation of E. F. Smith's ('20) description is presented:

A white, rapid-growing, non-sporiferous, Gram-negative, motile, peritrichiate-flagellate, promptly liquefying, nitrate-reducing, aerobic and facultative anaerobic, acid-forming, gas-forming, milk-curdling (by formation of an acid), dry-air-sensitive, rod-shaped or filamentous schizomycete, forming quickly on agar plates circular, grayish white, well-developed colonies; on very thin-sown gelatin plates characteristic, rapid-growing, big, circular, opaque white colonies. Organism white on most media but on Soyka's milk rice it is pale pinkish cinnamon. Bouillon clouded very quickly and gelatin stabs develop a prompt funnel of liquefaction. The organism does not form indol, and does not grow in Cohn's solution. It produces a non-volatile acid from dextrose, saccharose, lactose, galactose, and maltose, and small quantities of gas from inosit (muscle sugar), lactose, and mannite. Pathogenic to potato shoots. Index No. 5312-32120-?212.

Bacillus solanisaprus Harrison.—Summary given herewith is compiled from Harrison's ('07) original description:

Morphology: Bacillus of variable size. From 24-hour-old beef agar spores not seen; flagella peritrichic; organism Gram negative.

Cultural features: Growth in agar abundant, glistening, opalescent; persistent growth on potatoes; uniform growth in gelatin cultures, filiform liquefaction; strong growth in nutrient broth; ring in surface growth; clouding strong, fluid turbid, fine sediment; growth in gelatin colonies slow, round to elliptical; agar colonies round to lenticular, shiny, entire.

Physiology: Prompt coagulation of milk, extrusion of whey in 3 days, a few gas bubbles visible; litmus milk acid. Strong growth in Uschinsky's solution. In fermentation tubes gas was produced in bouillon containing mannite and lactose; growth in closed arm, with production of acid in each of the following: dextrose, saccharose, lactose, maltose, glycerin, mannite, and levulose. Nitrates in nitrate broth were reduced, nitrites present, indol production moderate to feeble; vitality on culture media long; thermal death point 54° C., optimum temperature for growth 25–28° C., growth slight at 37° C.; maximum temperature for growth 37.5°C., minimum temperature for growth about 0° C. Pathogenicity proven to following vegetables: potato, tomato, Jerusalem artichoke, and others; also to living plants of potato, tomato, common red pepper, and slightly in cucumber and physalis. Index No. 5312–32120–1212.

Bacillus melanogenes Pethybridge and Murphy.—The account presented below is summarized from Pethybridge and Murphy's ('11) original diagnosis:

Morphology: Vegetative cells 0.7–0.9 μ × 1.3–1.8 μ, found most frequently in pairs. Flagella peritrichic (fewer than in *B. solanisaprus* Harrison). No endospores. Gram's stain negative.

Cultural features: Nutrient broth clouded, more or less sediment, fluid turbid, no pellicle. Organism did not form a distinct ring on surface of potato juice. Gelatin colonies round. Growth best at top in gelatin tubes, some growth along needle track Gelatin liquefied. On potato plugs growth abundant, and chromogenesis yellowish.

Physiology: Facultative anaerobe. Milk acid curd, curd not very compact. No indol produced, nitrates reduced to nitrites with formation of gas. Diastatic action on starch. Acid and gas in fermentation tubes, in broth plus glucose, lactose, and saccharose. No acid from glycerin. Pathogenic to potato.

They comment upon the marked resemblance of their organism to *B. phytophthorus* Appel and state that they were tempted to regard it as a variety of the latter. Index No. 5312–3211?–1111.

After comparing the contributions of these earlier investigators, and in the light of the observations which I had previously made, I planned a thoroughgoing study of the relationship of the blackleg pathogen. To this end it was thought best to make careful comparative studies of a number of strains, including if possible the 4 species described above.

Source and history of the cultures used.—Some difficulty was experienced in obtaining authentic and viable subcultures of the organisms described by van Hall, Appel, Harrison, and Pethybridge and Murphy. Cultures of these organisms, however, were

finally obtained.[1] These were supplemented by cultures of the blackleg parasite isolated in Montana, Minnesota, and Maine. For the sake of convenience all of the subcultures collected were designated by a number. In order to facilitate matters, all records of studies made on the strains selected were kept under an assigned number.

Below is given a brief note on the source and history of the several cultures used more or less extensively throughout the writer's comparative studies. The first 5 enumerated were from isolations made in the Botany Department at the Montana Experiment Station and were selected from isolations made from potatoes affected with the blackleg disease obtained in some of the more important potato-growing sections of that state.

No. 160.1. Isolated 8/14/15, from tuber affected with black rot. Material from near Bozeman, Mont. Pathogenicity established. Still virulent late in 1917.

No. 170.3. Isolated 8/22/15, from tuber affected with black soft rot. Material from near Lewistown, Mont. Pathogenicity established. Still virulent late in 1917.

No. 180.2. Isolated 7/24/16, from black, cortical lesions on stem. Material from near Kalispell, Mont. Pathogenicity established. Still virulent late in 1917.

No. 183.2. Isolated 8/27/16, from black cortical lesions on stem. Tubers on same vine affected with blackleg rot. Pathogenicity established. Still virulent late in 1917.

No. 187B.1. Isolated 9/2/16, from tuber with superficial black rot lesions. Pathogenicity established. Still virulent late in 1917.

No. 191. Kindness of W. J. Morse. His "*B. phytophthorus* from Appel." Pathogenicity not established by the writer. Morse found it non-pathogenic.

No. 193. Kindness of W. J. Morse. His "III A," isolated by him in Maine, Aug., 1908. Found by the writer to be pathogenic. Still virulent late in 1917. Used by Morse in his studies ('17).

No. 194. Kindness of W. J. Morse. His "SE." Isolated by him in Maine, Aug., 1908, from a "potato stem showing a very rapid soft rot." Found by the writer to be pathogenic. Still virulent in late 1917. Used by Morse ('17).

[1] My thanks are especially due Dr. W. J. Morse and Dr. B. M. Duggar for very material assistance rendered in supplying certain of the cultures used in this work.

No. 195. Kindness of W. J. Morse. His "II P." Isolated by him in Maine, Aug., 1908, "from typical blackleg plants". Found by the writer to be pathogenic to potato tubers. Still virulent late in 1917. Used by Morse ('17).

No. 196. *B. solanisaprus* Harrison. Kindness of W. J. Morse, who used this strain in his studies ('17). The culture was originally procured from S. F. Edwards, Ontario Agr. Coll., in March, 1909. Found by the writer to be pathogenic to potatoes. Virulence in 1917 good.

No. 197. *B. atrosepticus* van Hall. Kindness of W. J. Morse. Previously studied by him ('17). This strain Morse "received under that name from Kral's laboratory[1] in 1910. . . . . At first it showed weak pathogenicity to potato tubers, and repeated inoculations to growing stems failed to produce the disease *until the present summer* [1916]." Found by the writer to be pathogenic, though, if anything, less strongly so than some of the others. Still virulent late in 1917.

No. 198. *B. melanogenes* Pethy. and Murphy. Kindness of W. J. Morse, who "received this from Dr. Pethybridge himself in 1911. . . . . It also has produced active decay of tubers and blackleg of the stem upon inoculation." Used by Morse ('17). I found it to be pathogenic. Still virulent in Nov., 1917.

No. 200. "*B. phytophthorus*" from Minnesota. Kindness of E. C. Stakman. Isolated 1915. The writer found it to be pathogenic to potato tubers. Still virulent in 1917.

No. 201. *B. phytophthorus* Appel. Kindness of B. M. Duggar who obtained the culture from E. F. Smith. The latter procured the strain from Dr. Aderhold in Berlin about 1906. It is presumed that Smith's culture was a transfer from Appel's original culture. The writer found it to be pathogenic. Still virulent in 1917.

No. 202. "*B. solanisaprus.*" Kindness of D. H. Jones who wrote that "the transfer purports to be from original strain of *B. solanisaprus* . . . . received in Oct., 1916, from the American Museum, to which Dr. Harrison had formerly sent a culture." Found by the writer to be pathogenic. Still virulent in late 1917.

II. Comparative Studies of Causal Organisms

Invigoration of cultures.—The cultures used by the writer were growing on beef agar at the time the comparative studies presented below were begun. Some had been previously cultivated on agar and some in nutrient broth for greater or less length of time. To begin with, all were plated out to insure purity of the culture and then each one was invigorated by trans-

[1] Kral's Bakteriologisches Laboratorium, Prague, Austria.

ferring to nutrient broth and cultivating at 25–27° C., on 3 consecutive days. All the cultures so treated responded promptly.

Pathogenicity.—Testing the pathogenicity of the strains to be used proved to be a considerable task. A number of trials were made before satisfactory proof was obtained of the pathogenic character of all of the strains in use. In the first series of tests potato tubers were used. On the whole, sound, smooth tubers of some thin-skinned variety, such as Early Ohio, provided the most satisfactory material for artificial inoculation experiments. Thick-skinned, late varieties, as the Russet Burbank, can be used. Tubers with a relatively high sugar content proved to be the best for this work (cf. Carbohydrate Utilization, page 45). On this account it is well if possible to obtain growing tubers, or else those from storage which are held till sprouts begin to develop.

Method.—Selected tubers were thoroughly washed by scrubbing in warm water, then soaked 3–4 hours in clean warm tap water, finally disinfected by immersing for 15 minutes in a 0.1 per cent aqueous solution of mercuric chloride. Immersion in this disinfectant for a longer period of time is not necessary. Moreover, it was found when the tubers were soaked for 1½–2 hours that the chemical remained in the outer layers of the skin, to the extent of making it difficult, if not impossible, to remove all of it by washing. Not infrequently the effects of a long soaking were manifest by inconspicuous lesions in the surface layers. Clean ground-glass plates, watch-glasses, and bell jars were made ready and sterilized in advance by washing in the bichloride solution. The tubers were then removed from the disinfecting solution, thoroughly washed in sterile tap water, and placed on watch glasses under the bell jars.

Preliminary experiments demonstrated the desirability of maintaining a nearly saturated atmosphere surrounding the tubers during incubation for 4 or 5 days. This requirement was easily satisfied by lining the inside wall of each bell jar with sterilized filter-paper wet with sterile water. If the atmosphere of the room is dry and cool it will be found best to place the inoculation chambers in an incubator kept at 22–25° C. All but one of the tubers under each bell jar were inoculated. Inoculations were made in a clean damp culture room. It was accomplished by placing a drop of the suspension from a recently in-

vigorated culture in a depression, usually near an eye, and stabbing through the inoculum 3 or 4 times with a sterile needle. The control tubers were pricked in similar places with a sterile needle. Inoculation courts were marked with an indelible pencil by tracing a good-sized circle around the spot where the tuber was pricked. Check courts were marked by a square.

Observations and conclusions.—The inoculation sets thus prepared were closely observed every day for 10 days, then with less frequency for over a month.

The number of days elapsing before signs of infection were visible varied, in the several experiments performed, from 2 to 6 days. Infection was in general more prompt where tubers having a high sugar content were inoculated, where the set incubated at around 23° C., and where the moisture content of the atmosphere surrounding the tubers was kept high. Observations made by the writer on the pathogenicity of several strains of the potato blackleg parasite suggest the conclusion that the "incubation period" in plants is markedly influenced by environmental conditions. The "incubation period" did not appear to be marked by a definite "turning point," as is the case in many animal diseases.

The writer concluded that all the strains being tested were pathogenic (cf. tables I and II). The nature and development

TABLE I

TABULAR SUMMARY

SOURCE AND HISTORY OF CULTURES USED

Cult. No.	Isolated	Source	Pathogenicity		Comparative virulence
			Stems	Tubers	
160.1	Tuber 8-14-'15	Montana	Marked	Strong	Average
170.3	Tuber 8-22-'15	Montana	Marked	Strong	Average
180.2	Stem 7-24-'16	Montana	Strong	Marked	Average
183.2	Stem 8-27-'16	Montana	Marked	Marked	Average
187B.1	Tuber 9-2-'16	Montana	Strong	Strong	Strong
193.	"Plant" Aug., '08	Maine, Morse's IIIA	Strong	Strong	Average
194.	"Stem" Aug., '08	Maine, Morse's SE	Yes	Yes	Average
195.	"Plant" Aug., '08	Maine, Morse's IIP	Strong	Yes	Average
196.	Before Mar., '09	*B. sol.* Ontario, Can.	Marked	Marked	Average
197.	Before 1910	*B. atro.* Kral's lab.	Marked	Yes	Average
198.	By P. & M. 1911	*B. mel.* fr. Pethy. Ireland	Yes	Yes	Low
200.	"Blackleg" 1915	Minnesota	No data	Yes	Low
201.	Before 1906	*B. phyto.* fr. Germany	Marked	Marked	Strong
202.	About 1907	*B. sol.* Am. Museum	No data	Strong	Average

of rot lesions in the tubers varied considerably under the different conditions of the artificial inoculation experiments. Lateral spread from the points of inoculation seldom took place to the extent it does in tubers invaded at the stem end and under natural conditions. The depth to which the lesions extended was usually to a point considerably beyond the depth of the needle prick, occasionally to the opposite side of the tuber. The character of the rot developed in the tubers inoculated artificially varied somewhat. Sometimes the affected tissues were quite moist and soft. Again, the tissues attacked were quite dry, being more or less spongy or cheesy in texture. Diseased material exhibited a more or less putrescent odor but in no case did inoculation with pure cultures effect a profound, gray, slimy, obnoxious, gaseous, soft rot. Variations, such as those mentioned, appeared to be associated to a considerable degree with (1) the available sugar content of the tuber, (2) the moisture content of the tuber, and (3) with certain environmental factors, notably temperature and humidity. The lesions in immature potatoes were generally more profound and the affected tissues softer than those in ripe tubers, particularly of a late, hard, starch-abundant variety A drop of inky black liquid was exuded at the points of inoculation in many cases. Occasionally a slight bulging of the tissues occurred at these points. When affected tubers were cut or broken open the diseased tissues were characteristically dark-colored, becoming brown or black upon exposure to the air.

Additional observations.—To extend the above observations on pathogenicity of the various strains at hand, the artificially inoculated tubers were cut into seed pieces and planted in pots in the greenhouse. In many instances the seed piece was entirely consumed by the blackleg rot before sprouts started. In a few cases, however, sprouts developed, only to be rapidly invaded by the pathogen and killed. The case of culture 160.1 is typical. Four stalks emerged from the ground but by the time they had grown to be from 3 to 6 inches tall (30 days after planting the seed pieces), the parasite invaded the stem tissues. The infection extended rapidly upward. At first the diseased tissues (cortical) appeared water-soaked. The tissues became dark-colored over night, and in a day or two the plants were prostrated (pl. 1, fig. 3). What proved to be a pure culture (plates) was recovered from this material. This was later used for inoculation work and found to be pathogenic in tubers.

Some experiments on the pathogenicity of the potato blackleg parasite performed at later dates furnish further proof of the pathogenicity of the strains under observation, and will be reported here. A brief survey of the results obtained in one of these experiments (II of 7/21/17) appears in tabular form below (table II). Tubers of the Russet Burbank variety were used. They had been kept in storage nearly a year. The tubers were washed and disinfected in the manner described previously. A number of selected tubers were then cut into seed pieces, each weighing 2 to 3 ounces. Three seed pieces were then inoculated with each of the several strains of the organism at hand. Two were planted in pots, the third being placed in a moist chamber kept at room temperature (20–24° C.)

TABLE II

PATHOGENICITY OF STRAINS OF B. ATROSEPTICUS EMPLOYED

Strain No.	Pathogenicity to seed pieces planted in soil	Pathogenicity to seed pieces kept in moist chamber
160.1	Positive (sprouts involved)	Negative (no infection, piece dry)
170.3	Positive (no sprouts)	Negative (no infection, piece dry)
180.2	Positive (no sprouts)	Positive (black rot)
183.2	Positive (no sprouts)	Positive (black rot)
187B.1	Positive (no sprouts)	Negative (no infection)
191.	Negative (sprouts)	Negative (non-pathogenic)
193.	Positive (sprouts)	Positive (black rot)
194.	Negative (sprouts)	Positive (black rot)
195.	Negative (sprouts)	Negative (no infection)
196.	Positive (no sprouts)	Positive (black rot)
197.	Negative (one sprout)	Positive (black rot)
201.	Positive (no sprouts)	Negative (no infection)

Evidently all the strains used were still virulent.

Among other things this experiment shows that the blackleg organism may destroy infected seed pieces before sprouts develop. In other cases the infectious material may be rendered inocuous or even killed by drying and by other agents. These conclusions are emphasized by experiments performed and reported above.

An experiment begun in August, 1917, showed that the strains previously listed were pathogenic to potato tops; that is, all introduced artificially gave rise to typical blackleg lesions in the stems and in leaf petioles.

A series of experiments was planned during the summer of 1917 in order to throw light on the question of whether potatoes planted in a soil contaminated with the blackleg parasite would

become affected with the disease. Only one trial was made, and while the results obtained do not in themselves warrant drawing a conclusion, they are of interest when compared with those reported by Shapovalov and Edson ('21). Ordinary seed pieces from sound, hard, healthy potatoes were planted in soil which had been previously heavily seeded with the blackleg germ by planting with tubers affected with the disease. The seed pieces sprouted and the plants therefrom grew to be of good size. No signs of infection by the blackleg germ were to be found in the seed piece, on the roots, or in the tops.

METHODS AND MEDIA

Certain of the bacteriological methods recommended by the American Public Health Association ('12) were followed as closely as possible by the writer in the prosecution of his studies of the cultural characteristics and physiology of the blackleg strains selected for comparison. In some cases this was not practicable or even possible on account of the fact that certain reagents were not available, due to conditions brought on by the World War. For this reason, a rather complete statement is given below of the media and methods employed, thus making it possible to duplicate both.

The present-day methods for determining H-ion concentration of bacteriological media were not in general use at the time the writer carried out his comparative studies. The ordinary media used were titrated with N/20 NaOH, using phenolphthalein as an indicator. In most cases the reaction of the medium was not adjusted. Wherever stated the reaction is given as "+ 7" etc., where the addition of 7 cc. of normal alkali per 1000 cc. would render the medium neutral to phenolphthalein. A "O" indicated that the medium as used was neutral to phenolphthalein. The precaution of having all flasks, beakers, test-tubes, etc. thoroughly clean was taken at all times.

Media.—Steps in the preparation of the media are briefly indicated below:

NUTRIENT BROTH

(1) To 1000 cc. distilled water add 50 gms. "Bacto" Beef Extract; (2) heat the mixture gradually up to 70°C. during about 1 hour; (3) boil for a few minutes to coagulate precipitable proteins; (4) make up the water lost by evaporation; (5) filter through layers of clean cheese-cloth; (6) cool to about 60°C. and add 1 per cent "Difco" peptone, ½ per cent NaCl, and dissolve; (7) filter through

paper; (8) titrate, using phenolphthalein as an indicator; (9) determine reaction and adjust if necessary; (10) sterilize in autoclave at 15 pounds for 15 minutes.

BEEF AGAR

(1) Nutrient broth (steps 1–6) is brought to a boil; (2) while boiling add 1 per cent "Bacto" agar, sifting it over the surface; (3) when agar is completely dissolved, cool and clarify in usual manner; (4) determine reaction and adjust if necessary; (5) filter; (6) sterilize in autoclave.

GELATIN

(1) To nutrient broth already prepared (steps 1–6), add 10 per cent "Bacto" gelatin and dissolve at a low temperature (60–70°C.); (2) determine reaction and adjust if necessary; (3) filter, tube, and sterilize at 15 pounds for 15 minutes.

DUNHAM'S SOLUTION

Make a paste of 10 gms. peptone and 5 gms. salt in a small quantity of water; then add sufficient water to make total used 1000 cc. Heat in flowing steam for ½ hour, then boil for 10 minutes over free flame. Make up water lost by evaporation. Filter through paper and tube. Sterilize in autoclave at 15 pounds for 15 minutes.

CARBOHYDRATE BROTH

(1) To nutrient broth already prepared (steps 1–6) add 1 per cent by weight of the chemically pure carbohydrate; (2) determine and record reaction; (3) filter, tube, and sterilize in autoclave at 15 pounds for 15 minutes.

MILK

The milk used was freshly drawn into a sterile bottle. After standing about an hour it was centrifuged and the cream skimmed off. Samples were titrated, using phenolphthalein as an indicator, and the reaction was determined to be "+20". The reaction was not adjusted. The medium was tubed promptly and sterilized by exposing tubes in flowing steam for 20 minutes on 3 consecutive days.

LITMUS MILK

To part of the fresh milk (see above), was added 2 per cent by volume of a saturated aqueous litmus solution (Merck's reagent, 1 gm. to 15 cc. H_2O). This medium was tubed immediately and sterilized by exposure to flowing steam for 20 minutes on 3 consecutive days. Both milk media were kept 4 or 5 days before being inoculated.

RAW POTATO PLUGS

A considerable number of raw potato plugs were prepared, observing all possible antiseptic precautions. The lot was kept several days in order to insure the removal of any contaminations that might appear.

RAW POTATO DISKS

Sterile disk-shaped slabs from raw potatoes were successfully prepared in the same manner as the preceding lot. They were placed in sterile Petri dishes on a piece of sterile wet filter-paper. On the whole, these disks proved to be more usable than the raw potato plugs.

COHN'S SOLUTION

Dissolve 5.0 gms. KH_2PO_4, 5.0 gms. $MgSO_4$, 10.0 gms. $(NH_4)_2$-$C_4H_4O_6$, and .5 gm. $(Ca_3(PO_4)_2)$ in 1000 cc. distilled water, and filter through paper. Sterilize in autoclave at 15 pounds for 15 minutes.

USCHINSKY'S SOLUTION (MODIFIED)

The modified solution was made up according to the formula given by Smith ('05).

EHRLICH'S INDOL TEST SOLUTION

Solution I.—Para-dimethyl-amido-benzaldehyde, 4.0 gms.; 95 per cent alcohol, 380 cc.; HCl (conc.), 80.0 cc.

Solution II.—Saturated aqueous solution of potassium persulphate.

To about 10 cc. of the liquid culture (preferably in Dunham's solution and 10 days old), add 5 cc. of Solution I, then 5 cc. of Solution II, using separate clean pipettes. Shake the mixture. The reaction may be accelerated by heating to about 70°C. The appearance of a red coloration which increases in intensity indicates the presence of indol.

NITRATE BROTH

Filter, tube, and sterilize in the autoclave 1 gm. peptone ("Bacto"), 0.2 gm. potassium nitrate cp. (Merck), and 1000 cc. distilled water.

REAGENTS AND TEST FOR NITRITES

Test for nitrites in nitrate broth culture on fifth day by adding: (1) one cc. of a 1 per cent potato starch water; (2) one cc. of a freshly prepared KI water (i.e., KI, 0.2 gm. in 50 cc. distilled water); (3) a few drops of strong H_2SO_4, (i.e., conc. H_2SO_4, 1 part, distilled H_2O, 2 parts). The appearance of a blue-black coloration in 10 to 15 minutes was taken as positive evidence of the presence of nitrites.

DETAIL OF COMPARATIVE STUDIES

MORPHOLOGY

A detailed study of the morphological characteristics of the several strains at hand was not attempted by the writer. However, it was determined that each was a small, short, actively motile, rod-shaped organism, easily stained by the ordinary aniline dyes. Pairs of the organism occurred in preparations made from fresh cultures on agar slants. No spores were found in any of the cultures, and all were determined to be Gram negative.

Cultural features and physiology.—In prosecuting the general plan outlined for making comparative studies of the cultural features and physiology, it was deemed wise to give particular attention and painstaking effort to the study of those items which are most commonly made use of in establishing bacterial species.

In recent times the response of the microorganism, as well as the reactions set up by it, in the presence of carbohydrates, has been stressed in attempts to distinguish between closely related species. On this account, and because of the fact that earlier students of the potato blackleg parasite report a great variety of conclusions as to the ability of this organism to ferment certain carbohydrates, the writer made exhaustive studies upon the gas- and acid-producing function of the blackleg bacillus. Details of the comparative studies appear on the several tables presented herewith and in the paragraphs below.

Nutrient broth:— Clouding prompt, persistent; medium becoming slightly turbid; sediment compact, granular, somewhat viscid; surface growth a ring, though under certain circumstances a light pellicle may form; no chromogenesis; odor absent; color of medium unchanged.

Earlier students of the blackleg bacillus did not wholly agree as to cultural features exhibited by this organism when grown in nutrient broth. The several strains studied were grown in 3 nutrient broths: (A) "Bacto" beef and "Bacto" peptone, (B) Liebig's beef extract and Witte's peptone, (C) fresh meat infusion and Witte's peptone, and all incubated at 27–28° C. All (except No. 191) were very similar in their response in nutrient broth. In those grown in "Bacto" beef and "Bacto" peptone (except Nos. 191 and 198) the surface growth was a ring, clouding was moderate, sediment granular-viscid, the medium slightly turbid, and no color. In the case of 191 the surface growth was a membrane, and in 198 a light pellicle with moderately strong clouding. When the strains were grown in Liebig's beef extract and Witte's peptone the surface growth was a ring (except No. 191 which was a membrane), clouding moderate, sediment granular viscid, medium slightly turbid, chromogenesis none. In those grown in fresh meat infusion and "Bacto" peptone, except Nos. 160.1 170.3, 187B.1, 191, 197, and 201, the surface growth was a pellicle, the clouding strong, the sediment granular-viscid, medium slightly turbid, and chromogenesis none. Nos. 160.1 and 187B.1 showed the surface growth a wide ring, and

moderate clouding; No 170.3 and 197, moderately strong clouding; No. 191, the surface growth a membrane with moderate clouding; and in No. 201 the surface growth was a light pellicle.

Observations recorded upon the cultural features exhibited by the different strains (except No. 191) as grown in these broths suggest a clue as to the disagreement between all who have cultivated the blackleg bacillus as to the character of the surface growth. It was found that the clouding was stronger and the surface growth heavier (a pellicle) in the nutrient broth made from a fresh meat infusion and "Bacto" peptone than in either of the other two nutrient broths used. Moreover, the growth response in the broth made with "Bacto" beef and "Bacto" peptone was somewhat more luxuriant than that in the beef extract and Witte peptone broth. The writer concludes that strain No. 191 is the only one of the 12 studied in broth that exhibits dissimilarity.

Agar stroke.—Growth moderate, filiform, somewhat raised, with smooth surface and glistening luster, translucent and somewhat iridescent, non-chromogenic; consistency butyrous, some quite viscid; odor absent; medium (color) unchanged.

On agar slants the cultural features were identical for the strains studied, except No. 191, where the form was spreading and effuse, the surface smooth, and luster dull.

Potato (cooked).—Growth moderate to abundant, filiform at first, spreading after a few days, slightly raised and flat, glistening, with a smooth to slightly contoured surface; chromogenesis yellowish white; odor not strong nor characteristic; color of medium slightly browned.

Special attention was devoted to the cultivation and study of the cultural features of the blackleg strains on sterilized potato plugs. This was done because of the importance attached by some authors to the cultural characteristics on this medium, in the differentiation of their "species" from others. All the strains studied comparatively by me were quite alike (except No. 191), in so far as can be detected from a study of the growth and cultural features on sterilized potato plugs. In No. 191 the growth was scanty, form spreading, surface smooth, dull, and colorless.

Potato (raw).—Raw potato slabs aseptically prepared as outlined in a preceding section were used. Preliminary tests indicated that infection and growth by the organism took place when the atmosphere of the culture chamber (Petri dish) was moist and when incubated at about 26° C.

The following strains were tested: Nos. 160.1, 170.3, 180.2, 183.2, 187B.1, 191, 195, 196, 197, 198. All responded and grew very similarly, except No. 191. In the case of the other 9 strains a considerable browning of the tissue approximate to the lines of inoculation was plainly visible at the end of the first day. A small quantity of dark brown to black liquid collected adjacent to the line of growth. By the end of a week abundant development of the cultures had taken place, forming a grayish white slime surrounded by a dark-colored border. The tissues at the margin of the growth were dark brown to black in color. Underneath the bacterial growth they were somewhat collapsed, so that the culture was growing in a shallow depression. No growth took place on the uninoculated slabs kept as controls.

Agar colonies (tubes inoculated at 28° C.).—Colonies rather small (3–15 mm. in 15 days); growth moderately rapid; form round to somewhat irregular; surface smooth; elevation raised and flat; edge entire, becoming undulate or lobed; internal structure, when magnified about 50 times, finely to coarsely granular; chromogenesis none; submerged colonies small, biconvex to ovoid, or nearly round; color of medium unchanged.

In strain No. 191, the colonies were large; surface smooth; elevation effuse; edge erose; and internal structure fine. This was the only one of the 11 planted in agar plates that developed markedly different features. In fact, one is led to the belief that no more than 1 species could possibly be represented by the 10 strains in question.

Gelatin colonies (incubated at 18–23°C.).—Form round to somewhat irregular; edge entire, often becoming undulate; liquefaction first noticeable, as a rule, about 24 hours after planting, proceeds rapidly, and is saucer-shaped; chromogenesis none.

Here again the cultural features and the physiological response as manifested by liquefaction were identical for all strains, except No. 191. In this case there was no liquefaction, and the colonies were larger. At about 21° C. colonies developed quite promptly in gelatin, and averaged 5–20 mm. in size, which was considerably larger than the agar colonies.

Gelatin stab.—Growth best at top; line of puncture filiform, often slightly beaded; liquefaction begins in about 24 hours and at 20°C., at first crateriform or napiform, becoming infundibuliform or stratiform, complete as a rule at end of third week, when culture is aerated by shaking occasionally; the liquid gelatin becomes cloudy and often a ring; sometimes a pellicle develops on the surface.

With a single exception (No. 191) the strains cultivated by stabbing gelatin plugs grew and responded almost identically as may be seen by glancing at table III.

Milk.—Fresh milk (+20) incubated at 28° C. is promptly coagulated. The amount of acid developed by the organism increases steadily for 18 days at least. Peptonization slow, begins in 2–3 days. In similar cultures to which litmus was added, a marked bleaching of the indicator took place by the end of the eighth day, as a rule. The addition of 2 per cent (by volume) of a saturated solution of Merck's purified litmus had no noticeable inhibitory effect upon the growth of the strains of the blackleg bacillus tested.

It may be noted that considerable difference appears in the descriptions published by earlier authors as to the cultural features and physiology of the blackleg bacillus when grown in milk. The writer failed to discover any real differences among the strains cultivated by him except in the case of strain No. 191 which, as has appeared heretofore, is non-pathogenic and otherwise quite different in its cultural features and physiology (see table III).

Uschinsky's solution.—Growth copious; fluid not viscid.

In this solution in which the nitrogen is supplied by organic compounds (see p. 28) all strains cultivated developed similarly except No. 191, which grew more copiously than the others with the development of a pellicle upon the surface of the medium.

TABLE III

CULTURAL FEATURES AND PHYSIOLOGY

GELATIN STAB* REACTION 0 INCUBATED AT 20° C

Culture	Growth	Line of puncture	Liquefaction		
			Type	Begins	Complete
				days	days
160.1	Best at top	Fil.-beaded	Strati.-infund.	2	22
170.3	Best at top	Fil.-beaded	Stratiform	1	22
180.2	Best at top	Fil.-beaded	Napi.-strati.	1	22
183.2	Best at top	Fil.-beaded	Napi.-strati.	1	22
187B.1	Best at top	Fil.-beaded	Crater.-strati.	2	22
191.	Best at top	Filiform	None		
194.	Best at top	Filiform	Strati.-infund.	1	22
195.	Best at top	Filiform	Strati.-infund.	1	22
196.	Best at top	Fil.-beaded	Napi.-strati.	1	22
197.	Best at top	Fil.-beaded	Crater.-strati.	2	22
198.	Best at top	Fil.-beaded	Napi.-strati	1	22
201.	Best at top	Fil.-beaded	Crater.-strati.	2	22

TABLE III—(Continued)

MILK	REACTION 20			INCUBATED AT 28° C.	
Culture*	Plain milk				Litmus milk
	Coagulation begins	Reaction		Peptonization begins	Reduction of litmus‡
		6 days	18 days		
	days			days	days
160.1	2	+23†	+47†	3	7-8
170.3	2	+33	+52	3	7-8
180.2	2	+36	+46	3	7-8
183.2	2	+34	+50	3	7-8
187B.1	2	+39	+49	3	7-8
191.	None	− 8	+24	None	None
194.	2			3	7-8
195.	2	+32	+53	3	7-8
196.	2	+34	+52	3	7-8
197.	2	+29	+39	3	8-9
198.	2	+10	+35	3	12
201.	2	+35	+51	3	7-8

* None of the strains changed the color of the medium.

† Figures represent increase or loss in degrees on Fuller's scale over controls titrated on same day.

‡ The indicator had very little, if any, effect on the growth.

Cohn's solution.—No growth.

Indol production.—Negative.

A survey of the published descriptions of the potato blackleg bacillus reveals the fact that half of the authors conclude that this organism produces indol, while the others arrived at an opposite conclusion. Special pains were therefore taken by the writer to determine this point accurately.

Kligler ('14), Lewis ('15), and others have criticized the use of the Salkowski-Kitasato method (conc. H_2SO_4 and $NaNO_2$) on account of its unreliability. Kligler ('14) states that this test should be discarded, since a red coloration is frequently obtained which is not due to indol; also because the reaction in cultures which really produce indol is not constant. Ehrlich's method of testing for indol production was used by the writer in his experiments. This test will detect indol in a dilution of 1:1,000,000. It has been shown that it is 10 times more delicate than the older test mentioned above. In order to make his tests even more searching, the writer employed both "Bacto" peptone and Witte peptone in making the Dunham's solutions used as culture media. All 12 strains of the blackleg bacillus were carefully tested, using controls inoculated with *Bacillus coli,* as well as blanks. A pink coloration did not appear in a single culture

except those in which *B. coli* was planted. Incidentally, it may be stated that the strain of *B. coli* used gave rise to a stronger color reaction in the Witte peptone solution than in the "Bacto" peptone solution.

Heating at 70° C. for a few minutes did not bring out the slightest trace of pink coloration in any of the cultures inoculated with strains of the blackleg organism. Cultures kept for 2 months were also tested, and negative results were obtained as before. In these cultures a yellowish or faint brown coloration was noticed after adding the test solution. Upon looking across the surface of the liquid against a dark background, the suggestion of a very pale wine coloration was obtained which in the absence of the positive test color might be mistaken for indol production. Possibly some of the authors who have reported that the blackleg bacillus produced indol were led astray by the appearance of similar color reactions in their test cultures. Repeated warnings have been given against accepting such colorations as a positive indication of indol. When compared with the pink color appearing in cultures of *B. coli* tested for indol, one is not likely to make an erroneous interpretation.

Nitrites from nitrates.— Nitrates reduced.

Strain No. 191 was the only one that did not reduce nitrates to nitrites. The starch-KI-sulphuric-acid test recommended by Smith ('05) was employed.

Ammonia production.—Moderate.

Standard nitrate broth was used as a culture medium. Duplicate tubes were inoculated, one of which was sealed with paraffin. When this was tested at the end of the tenth day, using Nessler's solution, a yellow coloration appeared in all tubes except controls. The color was slightly deeper in the sealed tubes.

Fermentation.—Acid and small volumes of gas from dextrose, lactose, sucrose, maltose, and mannite. No acid or gas from glycerin, dextrin, and potato starch. Moderate growth in closed arm of fermentation tubes, from which dissolved oxygen is driven off, in presence of dextrose, lactose, sucrose, and maltose. No growth at first, feeble growth later in closed arm under similar conditions, in presence of glycerin, dextrin, and potato starch. Quantitative consumption of common hexose sugars and of sucrose, lactose, and maltose. No hydrolysis of dextrin and potato starch.

As was stated in an earlier paragraph, the several students of the blackleg bacillus differ in their published observations and conclusions as to the gas- and acid-producing capacity of different isolations of this parasite. This fact is clearly shown in the tabular summary presented herewith (table VI).

The results of the writer's fermentation studies are briefly summarized in table IV. The data presented here lead to the conclusion that the several strains of the organism studied (except No. 191) are similar and that all produce small quantities of gas and considerable acid from dextrose, lactose, and sucrose, but no acid or gas from glycerin or potato starch.

In the course of his studies the writer determined that some of the strains at hand produced gas and acid from fructose, galactose, and maltose as well (compare with results of quantitative experiments on carbohydrate consumption).

Among other things, it was determined that the gas-producing function of the blackleg bacillus varies, depending upon (1) oxygen relations, (2) the length of time cultivated in the presence of a sugar, (3) the composition of the culture medium, etc., more than upon the particular strain or strains of the organisms under observation. In one experiment (Exp. "A", table V) only the minutest volumes of gas collected in the closed arm of 3 of the tubes (lactose) out of a total of 90 inoculated (30 of lactose alone). In this set 1 per cent of the carbohydrate was added to Dunham's solution (the reaction was not adjusted) and distributed in fermentation tubes. The tubes were heated in streaming steam until the air dissolved in the solution had been expanded and driven out. The bubble which collected in the top of the closed arm of the tubes was carefully tilted off before the set was autoclaved. Small bubbles found in the closed arm of 2 of the tubes upon removal from the autoclave were tilted off. Invigorated cultures were used for inoculation, and this was accomplished in the usual manner as soon as the culture medium was cooled. The cultures were incubated at 27–28° C. A feeble to moderate growth developed in the closed arm of all tubes containing glucose, lactose, and sucrose, also in tubes under observation containing fructose, galactose, maltose, but not in closed arms of tubes containing glycerin, dextrin, and potato starch. Under the circumstances it may be concluded that the strains of the blackleg bacillus under observation grow anaerobically only when they obtain sufficient

TABLE IV

PHYSIOLOGY (FERMENTATION)

Culture No.	Medium containing Dunham's solution and:															Controls
	Dextrose			Lactose			Sucrose			Glycerin			Potato starch			
	Gas	Reaction* 1 dy.	Reaction* 5 dys.	Gas	Reaction 1 dy.	Reaction 5 dys.	Gas	Reaction 1 dy.	Reaction 5 dys.	Gas	Reaction 1 dy.	Reaction 5 dys.	Gas.	Reaction 1 dy.	Reaction 5 dys.	Gas
160.1	+	+4	+ 8	+	+2	+7	+	+3	+ 8	0		0	0		−1	0
170.3	+		+10	+		+6	+		+10	0		0	0		0	0
180.2	+		+ 7	+		+8	+		+ 9	0		0	0		0	0
183.2	+		+ 8	+		+7	+		+ 6	0		0	0		0	0
187B.1	+	+4	+10	+	+4	+7	+	+5	+ 9	0	−1	1	0		0	0
191	0		− 8	0		−6	0		− 7	0			0			0
194	+		+ 8	+		+5	+		+ 6	0		0	0		0	0
195	+	+3	+ 8	+	+1	+7	+	+3	+ 5	0	−2	−2	0	−1	−1	0
196	+	+4	+12	+	+4	+8	+	+5	+11	0	−1	−1.5	0	0	−2	0
197	+	+5	+10	+	+1	+7	+	+3	Lost	0	−1	−1	0		−0.5	0
198	+		+ 8	+		+9	+		+9	0		0	0		−0.5	0
201	+	+8	+10	+	+4	+6	+	+4	+9	0	−1	−1	0	+1	0	0

* Reaction data represent gain (+) or loss (−) in degrees on Fuller's scale, over controls, after time intervals specified.

TABLE V

GAS PRODUCTION

Cult No.	Exp. A			Exp. B			Exp. C		
	Dex.	Lac.	Suc.	Dex.	Lac.	Suc.	Dex.	Lac.	Suc.
160.1	0	0	0	0	+	+	+	+	+
170.3	0	0	0	0	+	+	+	+	+
180.2	0	+	0	+	+	+	+	+	+
183.2	0	0	0	+	+	+	+	+	+
187B.1	0	+	0	+	+	+	+	+	+
191	0	0	0	*	*	*	*	*	*
194	0	0	0	+	+	+	+	+	+
195	0	0	0	+	+	+	+	+	+
196	0	0	0	0	+	+	+	+	+
197	0	0	0	+	+	+	+	+	+
198	0	+	0	+	+	+	+	+	+
201	0	0	0	+	+	+	+	+	+

* No data.

TABLE VI

DATA ON CARBOHYDRATE REACTIONS

Investigator	Dextrose		Lactose		Saccharose		Glycerin		Diastatic action
	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	
van Hall	+	+	0	+	+	+	0	0	0
Appel	*	*	*	*	+	*	*	*	*
Smith	0	+	+	+	0	+	*	*	*
Harrison	0	+	+	+	0	+	0	+	+
Pethy. & Murphy	+	+	+	+	+	+	0	0	+
Morse	+	+	+	+	+	+	0	+	+
Paine	+	+	+	+	+	+	*	*	+
Shapov. & Edson	+	+	+	+	+	+	0	0	0

* No data.

oxygen for metabolic processes from oxygen-containing substances which they can break down. It may be further concluded that the gas production is inhibited by the lack of free O_2.

In another set of tests (Exp. "B," table v) the same strains were employed as before, being cultivated in plain peptone water to which 1 per cent of the carbohydrate was added. The reaction was not adjusted. The Board of Health type of fermentation tube was employed, and sterilization was accomplished in the autoclave. After autoclaving, the tubes were set aside for a few days. Just before inoculation, the medium in each was thoroughly mixed by tilting the tube, but no free bubbles of air

were left in the closed arm. Inoculation was made in the usual manner, using invigorated strains. The cultures were kept at room temperature, 18–22° C. Gas production took place in all but 3 of the cultures (Exp. "B", table v) as was evidenced by the accumulation of from 1 to 8 or 9 per cent of a gas in the closed arm. Only very slight increases in the volume of the gas present occurred after the end of the first week. During the first 4 to 6 days there was just about as much growth in the closed arm of the tubes as in the open arm.

Still another test of the gas-producing capacity of the blackleg strains was made, using agar shake cultures. Small amounts of gas were produced by all the strains tested (Exp. "C", table v), as was evidenced by the development of a greater or less number of bubbles in the medium.

The appearance of gas bubbles in 1-day-old glucose agar slant cultures suggested the use of this type of test. A 0.75 per cent meat extract agar was used. The reaction of this medium was not adjusted. To this was added 1 per cent of the carbohydrate to be investigated. The medium was melted, cooled to 42° C., and inoculated with 3-mm. loops of invigorated broth cultures. The inoculum was thoroughly mixed and the medium aerated by a vigorous rolling between the hands. The cultures thus made were set aside in a vertical position to cool and were incubated at 27–28° C. In this type of culture it was found that there was little increase in the volume of gas produced later than the second day after inoculation, as evidenced by the number and size of the bubbles.

In following up this line of experimental work it was later determined that when agar shake cultures were sealed with paraffin a somewhat greater volume of gas was demonstrable, as evidenced by the increased number and size of the bubbles in the medium. What is even more significant: It was found that the cultivation of a particular strain (or strains) in the presence of a certain carbohydrate increased its powers to ferment that carbohydrate with the production of gas. In this way certain strains which at first produced gas very weakly, if at all, were "trained up" to ferment the particular carbohydrate in question more vigorously, as was evidenced by the production of larger and larger volumes of gas.

Diastatic action.—Absent.

The writer's conclusion is based upon the results of carefully conducted quantitative determinations of carbohydrate hydrolysis by certain of the blackleg strains at hand. In this phase of the work the hydrolysis of potato starch, as well as other carbohydrates, was investigated. No evidence was found of the slightest hydrolysis of potato starch by the blackleg bacillus after an interval of 6 days. The ordinary tests for diastatic action were made, using a nutrient agar starch jelly, and these yielded no evidence of diastatic action. These results are not in accord with those of most investigators (table vi).

Active acidity and titratable acidity of culture solutions.—While the greater part of the experimental work reported upon here was done in 1916 and 1917, the data presented in this section were obtained in May, 1922. Experiments were made in order to determine the production of acid, as shown by the determination of H-ion concentration, as well as by the relative amount of titratable acid produced by *Bacillus atrosepticus* when cultivated in the presence of certain sugars. Subcultures of strain No. 196 were employed in these experiments.

To a nutrient broth made as before 1.0 per cent of the following sugars was added: (a) glucose ["Difco"], (b) sucrose [Merck's highest purity], (c) lactose [Merck's].

Cultures and methods.—Comparatively large-volume cultures were employed, 500 cc. of each of the sugar broths being placed in each of 3 Florence flasks of 1 liter capacity. Sterilization was accomplished in the autoclave at 15 pounds pressure for 15 minutes. In order to avoid the lag phase of growth (Chambers, '20) a rejuvenated broth culture 7 hours old was used for inoculating the sugar broth employed.

The H-ion concentration of each culture medium was determined at the time of inoculating the sugar broths and thereafter as indicated (table vii). The colorimetric method of Clark and Lubs ('17) was employed. The production of titratable acid was determined in the usual manner, using 0.507 NaOH and phenolphthalein, and the amount of titratable acid was expressed in degrees on Fuller's scale where "+10"=0.1 of 1 per cent normal HCl.

Buffer index.—Following the method of Brown ('21) the buffer index of the medium was determined in order to provide

additional data for comparison if need should arise. All 3 sugar broths employed were found to have a buffer index of 1.5 where "BI" is the sum of the reserve alkalinity and the reserve acidity, each value being expressed in per cent normal alkali or acid.

TABLE VII

H-ION CONCENTRATION AND TITRATABLE ACIDITY FOR STRAIN NO. 196

Medium	Cultures									
	Controls		1 day		2 days		5 days		11 days	
	P_H	Titr. acid.	P_H	Titr. acid.	P_H	Titr. acid.	P_H	Titr. acid.	P_H	Titr. acid.
Glucose	6.0	+16	5.4	+20	5.2	+22	5.0	+25	5.0	Not determined
Sucrose	6.0	+16	5.4	+21	5.0	+24	5.0	+25	5.0	Not determined
Lactose	6.0	+16	5.6	+20	5.2	+21	5.2	+25	5.1	Not determined

From table VII it may be noted at a glance that (1) some acidity is developed by *Bacillus atrosepticus* when grown in the presence of certain sugars, and (2) essentially equivalent amounts of acid are developed in the presence of all 3 sugars employed.

Attention is also directed to the fact that the major increase in H-ion concentration took place during the first 2 days, and it is believed (see Chambers, '20) that a true acidity of about P_H 5 inhibits further growth and reproduction of the organism.

SUMMARY OF COMPARATIVE STUDIES OF CAUSAL ORGANISMS

The salient characteristics of the several strains of the blackleg bacillus studied by the writer are brought together in compact form below. The scheme adopted is based upon the Descriptive Chart indorsed by the American Society of Bacteriologists in December, 1920. The digits used to designate the several "primary" and "secondary" characteristics of the strains in question may be translated in detail by reference to the foregoing text, and to the "brief characterization" column upon the descriptive chart above mentioned. The data below indicate the fact that there is very little, if any, difference between the organisms studied by the writer, except No. 191, which aside from being non-pathogenic to the potato, differs from the others in many important respects.

BRIEF CHARACTERIZATION

160.1	Montana strain	53*2-32120-2111-2222- *211-22-121
170.3	Montana strain	53 2-32120-2111-2222- 211-22-121
180.2	Montana strain	53 2-32120-2111-2222- 211-22-121
183.2	Montana strain	53 2-32120-2111-2222- 211-22-121
187B.1	Montana strain	53 2-32120-2111-2222- 211-22-121
191.	*B. phytophthorus* "as rec'd. from Appel."	53 -51230- 333-1122- 322-33-322
194.	Maine strain Morse's "SE"	53 2-32120- 111-2222- 211-22-121
195.	Maine strain Morse's "IIP"	53 2-32120-2111-2222- 211-22-121
196.	*B. solanisaprus* Harrison	53 2-32120-2111-2222- 211-22-121
197.	*B. atrosepticus* van Hall	53 2-32120-2111-2222- 211-22-121
198.	*B. melanogenes* Pethy. & Murphy	53 2-32120-2111-2222- 211-22-121
201.	*B. phytophthorus* Appel	53 2-32120-2111-2222- 211-22-121

While all differences, great or small, have been recorded, it is significant that these differences, except in the case of No. 191, are insufficient to appear in the brief characterization of each strain set down above. Such variations as may exist are, it seems to me, wholly insignificant and do not justify even varietal characterization. Within the limits of his comparative studies Morse ('17) reached similar conclusions. Among his cultures were some of the same strains used by the writer, namely, Nos. 194, 195, 196, 197, and 198.

RELATIONSHIPS AND NOMENCLATURE

Differences between the 4 "species" of the blackleg bacillus as originally described may be noted by referring to the original descriptions or to abstracts prepared by the writer (see p. 17 et seq.).

The index numbers brought together below assist in visualizing the small differences upon which certain of these "species" were established.

B. atrosepticus van Hall (van Hall, '02)......5312-32120-2121
B. phytophthorus Appel (Appel, '03) ....5?1?-32120-???1
B. phytophthorus Appel (Smith, '10).........5312-32120-?212
B. solanisaprus Harrison (Harrison, '07) 5312-32120-1212
B. melanogenes Pethy. & Murphy (Pethybridge and Murphy, '10) 5312-32110-1111

* Omission of figure indicates item not determined.

Reference to the literature discloses the fact that Appel ('02, '02a) succeeded at an early date in isolating a bacterial organism which he believed to be the cause of the potato disease in question. The motive which prompted him to publish brief notes (Appel, '02, '02a) on the disease, and at the same time to propose a name (only) for the causal organism, must be left for conjecture. The fact remains that he (Appel, '03) did not publish a full description of either the organism or the disease until about a year after van Hall's ('02) dissertation was available to science.

Pethybridge and Murphy ('11) must have recognized that their species was very similar to van Hall's and Appel's, for in regard to "*B. phytophthorus*" and "*B. melanogenes*" they state: . . . "the two organisms, if not identical, are at any rate closely allied; and it is perhaps with some reluctance that we regard it as a distinct species and suggest the name *Bacillus melanogenes* for it." Relative to *B. atrosepticus* van Hall, these authors say that it has some points in common with their species, but differs by "occurring chiefly as isolated individuals, whereas ours is more frequently found in pairs. The former is also decidedly smaller in size, in spite of variations in both cases, and its action on milk appears to be different from that found in our case. *We were unfortunately unable to obtain a copy of the detailed character of B. atrosepticus* [italics not in original] before our own work was concluded."

Harrison ('07) states that "the symptoms of the Ontario disease somewhat resemble those described by Appel for the 'Schwarzbeinigkeit,' a disease which seems rather widespread in Germany." Further than this, the only comparisons made by him appear in a little display chart on page 592 of his paper. In this exhibit certain of the morphological and biological characteristics of "*B. phytophthorus*", "*B. atrosepticus*," and "*B. solanisaprus*" are compared. The differences brought out are, it seems to me, more apparent than real, as shown by the index numbers compiled (see p. 41).

As was pointed out in an earlier paragraph, Appel's name "*Bacillus phytophthorus*" appears as a *nomen nudum* in a brief paper published by him about two months previous to the appearance of van Hall's "*Bacillus atrosepticus.*" Furthermore, van Hall's dissertation embodies the earliest published description of the potato blackleg disease which is sufficiently definite

and detailed to enable one to identify the disease ascribed to the bacterium named and described as the cause.

For a number of reasons Smith ('20) believes it best to retain Appel's name, especially as van Hall made very few inoculations under natural conditions, and further, because he says of his organism: "On artificial media the parasite loses its virulence very quickly." This contention loses weight in the light of a similar statement made by Smith on a preceding page (see Smith, '20, page 263), as follows: "It is common belief *(of German origin)* [italics not in original] that the organism loses virulence readily." The writer feels that Smith's further objections to the use of van Hall's name are largely refuted by the data and facts previously presented.

It was inevitable that questions involving nomenclature should have appeared in this paper and a serious attempt has been made to give all names most careful consideration. On the grounds of priority as well as for other reasons brought out above the writer believes that *Bacillus atrosepticus*[1] van Hall should stand.

REVISED DESCRIPTION OF BACILLUS ATROSEPTICUS VAN HALL

Index No. 5312-32120-2111.[2]

Microscopic features.—The potato blackleg bacillus is a small non-sporiferous, Gram-negative rod, having an average diameter of about 0.6 μ and a length (1.5 μ) slightly exceeding twice its diameter. The organism is actively motile by means of a few peritrichic flagella. No capsule is demonstrable by the ordinary methods now in use.

Physiological characteristics and cultural features.—The organism is non-chromogenic in agar, gelatin, nutrient broth, and other common media. On agar slants growth is moderately abundant. The surface is smooth and the luster is glistening. Agar colonies are small, round to somewhat irregular in form, and, under a magnification of 50 diameters, they appear to be granular in structure.

Gelatin is liquefied, the action being visible on the second day, if not before. Colonies on gelatin plates are white, round, and noticeably larger than those which develop on agar.

[1]According to a recently suggested outline of bacterial classification (Winslow et al, '20) the name would become *Erwinia atrosepticus* nov. comb.

[2]See Descriptive Chart indorsed by Soc. of Am. Bacteriologists, Dec. 30, 1920.

Nutrient broth is promptly clouded, the medium becoming slightly turbid after a few days. The characteristic surface growth is a ring, though in some broths a light pellicle may develop.

Milk is promptly coagulated, the amount of titratable acid produced by the organism increasing steadily for several days. A slow peptonization of the curd begins on the second or third day.

In Cohn's solution there is no growth. In Uschinsky's solution the growth is copious, but the fluid does not become viscid.

No indol is demonstrable by the Ehrlich test, in either young or old cultures grown in peptone water made with (1) Witte peptone, or (2) Bacto peptone. Nitrates in nitrate broth are reduced to nitrites without the formation of gas. Ammonia production is feeble to moderate.

Carbohydrate reactions.—Acid and small volumes of gas are produced from dextrose, galactose, sucrose, maltose, and mannite. The gas-producing capacity is not particularly characteristic. No acid and no gas are produced from glycerin, dextrin, and potato starch. Quantitative consumption of the common hexose sugars, glucose, fructose, and galactose, has been demonstrated. Likewise, the organism consumes sucrose, lactose, and maltose. A sugar concentration of 0.25 per cent is ample. Diastatic action is absent, both in respect to starch and dextrin.

Enzymes.—This organism secretes several carbohydrate enzymes, as is shown by its action on as many different saccharides; cytase also is probably produced.

The optimum temperature for growth is about 26° C., with no growth at 37.5° C. The organism withstands considerable extremes of cold, being found viable in soil cultures exposed for 24 hours to temperatures ranging from —6.7° to —28.2°C.

The blackleg bacillus is quite resistant to drying and remains viable for long periods of time on plain beef agar.

Virulent strains produce a necrosis of the stem and tubers of the potato. Parenchymatous tissue is almost exclusively affected, and a blackening of the diseased tissues is characteristic.

III. Carbohydrate Utilization by Strains of the Blackleg Bacillus and Other Microorganisms

In spite of the fact that the strains of the blackleg bacillus employed by the writer in earlier studies showed no signs of diastatic action on potato starch jelly, it was nevertheless somewhat difficult to conceive of a virulent parasite of the potato totally lacking the power of hydrolyzing starch. This thought, together with a rather extensive knowledge of the gas- and acid-producing capacity of this bacillus, suggested the desirability of investigating these relations in a quantitative manner. The writer had employed Shaffer's ('14) modification of the volumetric method of Bertrand for the quantitative estimation of reducing sugars in the presence of proteins and albumins. Familiarity with this method led me to conclude that with its aid reliable data could be obtained concerning the quantitative consumption of certain sugars and starch by the blackleg bacillus as well as by other microorganisms with which it appeared. Very little, if anything, had been published upon the quantitative consumption of carbohydrates. Accordingly, plans were made to investigate quantitatively these relations in certain of the strains of the potato blackleg bacillus as well as in other species of bacteria. Some points of interest in this connection have, however, recently been contributed by Besson, Ranque, and Senez ('19). Working with *Bacillus coli* in nutrient broth containing varying amounts of dextrose, they found that when less than 0.4 per cent dextrose was furnished all the sugar was removed in 24 hours.

MATERIALS AND METHODS

After numerous experiments a method was developed whereby reliable data were obtained. The work of Shaffer and Hartmann ('21) on the iodometric determination of copper and its use in sugar analysis had not appeared at the time the writer performed the experiments reported herewith. Undoubtedly, the newer methods would have greatly facilitated prosecution of this phase of the work, but it is doubtful if more accurate results could have been obtained.

Certain strains of the blackleg bacillus used in the preceding studies were selected for investigation and comparison in this connection, i. e., Nos. 160.1, 180.2, 183.2, 187B.1, 195, 197, 198, 201. Pure cultures of the following were also tested: *Bacillus coli*

communis, B. vulgaris, and a species of yeast isolated from a compressed yeast cake.

The basic culture solution used throughout was a Dunham's solution to which 1 per cent of the following test substances (Merck's, except starch) was added: (1) glucose (H. P.), (2) fructose (cr.), (3) galactose (powder), (4) saccharose (H. P.), (5) lactose (H. P. cr.), (6) maltose (powder), (7) dextrin (H. P. powder), and (8) potato starch (Heil's).

The nutrient solutions used were not adjusted by the addition of acid or alkali. As a matter of fact, it was determined in a preliminary experiment that if small amounts of acid (HCl) were added to adjust the reaction a considerable hydrolysis of certain disaccharide sugars resulted during the process of sterilization, regardless of whether the latter was accomplished in the autoclave or in the Arnold sterilizer (table IX). The Dunham's solution employed as a basis of the nutrient solutions used was found to be P_H 7.0, approximately. The P_H of the several sugar broths employed was not determined, but it is unlikely that they differed markedly in H-ion concentration from the Dunham's solution.

Invigorated cultures of the several microorganisms at hand were employed. Ten-cc. volumes of the media were measured into special culture tubes, called wasp tubes (see pl. 2, fig. 5). (The wasp tube was suggested to me by Dr. G. W. Freiberg, formerly of this laboratory, and has greatly facilitated and expedited my work.) These were plugged in the usual manner and sterilization was accomplished in the autoclave (see table IX). Duplicate cultures were made, and a number of controls were carried along with each series of cultures. All cultures were incubated at 28° C. for 6 days, as it was found that there was no point in carrying the cultures for a longer period. Chambers ('20) has recently contributed a convincing explanation of why evidences of the metabolic activities fail after a few days in certain sugar-broth cultures.

Shaffer's ('14) method for the quantitative estimation of reducing sugars was used with slight modifications. This development of the permanganate titration method of Bertrand is of special value to workers in plant physiology because it enables one quickly and accurately to determine the amount of sugar as low as 2 mgms. The copper-reducing value of the culture

medium in which the microorganisms in question were grown was determined by this method, as was the copper-reducing value of control portions. Differences were carefully noted and carbohydrate utilization estimated by reference to Shaffer's table of copper-glucose equivalents, having first determined by calculation that 1 cc. of the N/20 permanganate is equivalent to 3.18 mgms. copper. For the technique of the complete method see under "Technique."

According to Plimmer ('15) pure lactose reduces Fehling's solution 71 per cent as strongly as glucose; pure maltose 64 per cent as strongly as glucose. These figures were employed in making estimates of the amounts of these disaccharides consumed by the microorganisms under consideration. It was assumed in this work that the fructose and galactose used reduced Fehling's quite as strongly as glucose. Certain dextrins reduce Fehling's slightly, but the product which I employed did not; nor did the saccharose and potato starch. Where it became necessary to hydrolyze these last-named substances in order to determine whether there had been utilization of the substance as such, the following methods were used:

Sucrose.—Davis and Daish ('13) employed citric acid in preference to a mineral acid for inverting cane sugar. In dealing with plant extracts, in which there was an accumulation of sodium acetate, they employed 10 per cent citric acid.

Method.—(1) Make solution faintly acid to methyl orange by addition of a few drops of concentrated H_2SO_4; (2) add 5 per cent by weight of citric acid; (3) autoclave for 15 minutes at 15 pounds pressure, cool, and neutralize to phenolphthalein with NaOH.

Starch and dextrin. Method.—(1) To a 1 per cent starch suspension (10 cc.) add 0.3–0.5 cc. conc. HCl and 10 cc. distilled water; (2) heat in autoclave for 15 minutes at 15 pounds pressure; (3) cool, neutralize to phenolphthalein with NaOH, and estimate as glucose. The amount of glucose times 0.9 equals the weight of starch hydrolyzed.

Maltose and lactose. Method.—(1) To 10 cc. of a 1 per cent solution of the sugar add 0.3–0.5 cc. conc. HCl (sp. gr. 1.16) and 10–15 cc. distilled water; (2) heat in autoclave for 15 minutes at 15 pounds, cool and neutralize to phenolphthalein by addition of NaOH; (3) dilute and estimate as glucose.

Complete reduction of these sugars is difficult, if not impossible, to accomplish by boiling for 60 to 90 minutes. Ordinarily, inversion is complete to the extent of 96–97 per cent. Heating for a longer time will accomplish more inversion, but is offset by an increasing destruction of the resulting monosaccharides.

A trial of the method developed by Ling and Rendle ('05) showed that this could not be used because of (1) a clouding of the culture solution immediately upon addition of the Fehling's, making it practically impossible to observe discharge of blue color upon nearing the end point, and (2) the CuO_2 does not settle readily. The trouble is probably due largely to the amino acids of the peptone in the Dunham's solution employed (cf. Davis and Daish, '13).

REAGENTS AND SOLUTIONS

Fehling's solution.—It was found best to use the Soxhlet-Fehling solution, preliminary tests having demonstrated that the solution as modified by Allihn contained too much alkali, when made up with NaOH at the rate of 178 gms. per liter.

Cuprous oxide solvent.—The ferric sulphate-sulphuric acid-cuprous oxide solvent recommended by Shaffer ('14) was employed. This solvent contains 10 per cent ferric sulphate, $Fe_2(SO_4)_3$, in 25 per cent sulphuric acid. It is a very active solvent, and care must be taken in watching for the end point. To be efficacious it should be made up by mixing equal parts of a 20 per cent aqueous ferric sulphate and 50 per cent sulphuric acid. The ferric sulphate should be dissolved in hot water, filtered while hot, and the warm acid mixed with the warm ferric sulphate solution. Just enough permanganate should be added to oxidize any ferrous salt which may be present.

Potassium permanganate solution.—The potassium permanganate used was made up according to Olsen ('10). Freshly prepared and carefully standardized N/20 permanganate was employed for titration. One cc. of N/20 permanganate is equivalent to 3.18 mgms. of copper.

TECHNIQUE

The determination of reducing sugars.—The following technique was followed in determining amounts of reducing sugars in carbohydrate broth cultures of bacteria. Certain slight modi-

fications of Shaffer's ('13) original method appear, but these are largely omissions of certain steps which were determined by preliminary experimentation to be unnecessary. For instance, acetic acid and colloidal iron were not used in the process because it was determined that the small amounts of albuminous material present in the cultures did not interfere with the determination of the reducing sugars present. The precautions emphasized by Shaffer were observed throughout. The cuprous oxide was titrated immediately upon being dissolved and without removal from the centrifuge tube in which it was precipitated and thrown down. In order to avoid breakage of the centrifuge tubes when these were plunged in the water bath circular wire baskets (pl. 2, fig. 5) having wooden bottoms and tops, with holes for 2 centrifuge tubes, were used. No oxidizable substance other than the sugar was allowed to get into the solution, and the cuprous oxide solvent employed was made from chemically pure substances in order to avoid presence of ferrous iron. This was further assured by adding a trace of permanganate.

Procedure.—The procedure outlined in the steps given below was followed in carrying out all experiments in this phase of the work:

(1) A large volume of Dunham's solution was made up, autoclaved, and then filtered through paper.

(2) To separate portions of this solution, 1 per cent of the test substances (see list p. 46) was added and dissolved.

(3) Exactly 10 cc. of each of the 8 nutrient solutions thus prepared were placed in each of 10 special culture tubes ("wasp" tubes, pl. 2, fig. 5). These tubes were large enough to permit dilution of the culture to exactly 60 cc. This was made possible by carefully standardizing each tube and permanently etching the 60-cc. mark on the slender neck. Had these tubes been drawn out and graduated to hold 50 cc., calculations would have been simplified.

(4) The tubes were plugged with cotton in the usual manner and the contents sterilized in the autoclave by exposing for 15 minutes at 15 pounds pressure. One of the preliminary experiments performed showed that this method, if carefully controlled, did not hydrolyze the di- and polysaccharides used (table IX).

(5) Each of the 8 media was inoculated with a loopful of an invigorated culture of the organism to be tested for its ability to hydrolyze the test substances employed. Two uninoculated "control" tubes of each sugar broth were carried along, incubated, and tested in identically the same manner as the inoculated cultures in each set.

(6) The sets thus made ready were incubated at 27–28°C. for 6 days, preliminary tests having shown that there was no point in incubating the cultures for more than 5 or 6 days (cf. Chambers,'20).

(7) The culture medium in each "wasp" tube was next diluted 6 times by adding distilled water to the 60-cc. mark. The contents of each was thoroughly mixed.

(8) Next, exactly 10 cc. of the diluted culture medium were removed and placed in a 50-cc. lipped centrifuge tube (pl. 2, fig. 5), which was marked to correspond to the culture tube from which the medium came. These 10-cc. volumes, then, represented 1/6 of the original culture solution, i.e. 1.666 cc. of the original 10; and if the 10 cc. of the culture solution contained 0.1 gm. of a carbohydrate a 10-cc. sample from the diluted culture should contain 0.0166 gm. of the substance provided none had been consumed.

Each tube in the set was handled in exactly the same way, the same pipette being used throughout.

(9) Ten cc. of freshly mixed Soxhlet-Fehling's solution were promptly added to the 10-cc. volumes in the centrifuge tubes.

(10) All were quickly placed in the special baskets and immersed in a water bath (pl. 2, fig. 5) where they were exposed at the boiling point for exactly 10 minutes.

(11) Upon removal from the bath the tubes were nearly filled with distilled water, pairs balanced, and centrifuged[1] at a moderate speed for 3–4 minutes.

(12) Upon removal from the centrifuge the Fehling's was cautiously decanted over a white dish, the cuprous oxide at the bottom being disturbed as little as possible, for fear of loss.

(13) This having been done each tube was again filled with distilled water in order to wash the precipitate, pairs balanced, and again centrifuged for about 4 minutes.

(14) When finally removed from the centrifuge the wash water was decanted as completely as possible without disturbing the cuprous oxide in the bottom (if any was present).

(15) The cuprous oxide was then promptly dissolved in as small a volume of the ferrous sulphate-sulphuric acid solvent as possible.

(16) The copper was then titrated immediately against N/20 potassium permanganate. No attempt was made to remove the dissolved cuprous oxide from the centrifuge tube, the actual amount of copper present being determined by titrating directly into the tube.

(17) Using the figures thus obtained and remembering that the 10-cc. sample titrated represents 1.666 cc. of the original culture medium,

[1] The No. 1 centrifuge of the International Instrument Co. was used.

the actual amount of sugar (as glucose) left was calculated by reference to Shaffer's ('14) table of copper-glucose values. In order to determine consumption of the non-reducing carbohydrates in use, i.e., sucrose, dextrin, and starch, it was necessary to accomplish inversion by heating in the presence of an acid (see "Methods," p. 47, et. seq). This having been accomplished, the procedure was exactly as outlined above.

The control tubes containing the several carbohydrate broths were handled in exactly the same manner as the cultures, except for inoculation, and were a component of each experiment.

EXPERIMENTAL DATA

The data relative to carbohydrate utilization are presented in tables IX–XIII. Where practicable the amounts of the test substance consumed are given in mgms. of the carbohydrate supplied. The most complete data are, for obvious reasons, given in terms of mgms. of glucose. The writer believes that the figures are reliable to the extent of showing relative differences in carbohydrate utilization as well as the amounts of the test substances consumed by each of the several species and strains of the microorganisms employed.

A slight error in the sugar data presented may have developed, due to the fact that it was necessary to make interpolations between figures available in tables IX–XIII, in order to determine the sugar equivalent of the copper values obtained from my own permanganate titration data. The following figures and statements are presented to illustrate the methods and the calculations employed in obtaining the sugar values presented in the above-mentioned tables. The specific case of *Bacillus coli* in the presence of glucose is selected for this illustration. The 10-cc. sample of the culture taken for treatment with Fehling's and subsequent titration represented (as in all other cases) 1.666 cc. of the original 10-cc. sugar broth in which the organism was cultivated. It was not necessary to account for possible loss due to evaporation, since the cultures were made up to 60 cc. before sampling.

Having determined the number of cc. of permanganate for 1 cc. of the original culture solution as 4.15, then the number of mgms. of copper is represented by 13.2, determined by multiplying 4.15 by the factor 3.18 (it was previously determined that 1 cc. of N/20 permanganate is equivalent to 3.18 mgms. copper).

Next, the glucose equivalent of the 13.2 mgms. copper is estimated by interpolating in Shaffer's table of copper-glucose equivalents, and finally, the amount of glucose consumed or used by the organism is found by determining the difference between the amounts of glucose recovered in the control and in the culture.

Where amounts of sucrose are given they were determined as follows:

$$96 : 100 :: A : X,$$

where the reducing ratio

$$\frac{\text{Glucose}}{\text{Invert sugar}} = 0.96,$$

and A is the invert sugar ("glucose") value obtained by titration and calculation, and X equals the true glucose value.

Then:

$$360 : 342 :: X : Y,$$

where 360 is the molecular weight of 2 molecules of $C_6H_{12}O_6$ and 342 is the molecular weight of $C_{12}H_{22}O_{11}$. Then, where X equals true glucose value determined above, Y equals sucrose consumed. Fructose and galactose consumption were calculated from the "glucose" values obtained, using the reducing ratios of these sugars as given by Browne ('12):

$$\text{Ratio } \frac{\text{Glucose}}{\text{Fructose}} = 0.92,$$

$$\text{Ratio } \frac{\text{Glucose}}{\text{Galactose}} = 0.90.$$

Consumption of lactose and maltose is directly determinable, since both these sugars are reducing sugars. Consumption of lactose, maltose, dextrin, and starch, as glucose, was determined by hydrolyzing portions of both control and culture solutions (see "Methods," p. 47). For well-known reasons no attempt was made to calculate consumption of dextrin and starch as such, nor was it thought to be worth while to convert the galactose-glucose value obtained by titrating the hydrolyzed lactose into glucose. It should be borne in mind that the glucose values given for dextrin and starch were derived by multiplying the glucose value obtained by titrating the hydrolyzed product by 0.9. Without doubt the figures in the tables representing consump-

tion are slightly erroneous, since, as is well known, more or less destruction of hexose sugar takes place in the process of hydrolyzing carbohydrates by heating in the presence of an acid. It may be recalled, too, that variability in the reducing power of lactose and of maltose is a characteristic of these disaccharides. According to Browne ('12, p. 402), succeeding portions of lactose and maltose will vary in reducing power according to the amount of free alkali, time of boiling, etc. "This peculiarity of maltose and lactose" he says, "is explained by a slight hydrolysis of the sugar into monosaccharides of higher reducing power" during the process of heating. Browne believes that a slight inversion of this kind takes place to a greater or less extent with all higher saccharides (including sucrose) upon boiling with Fehling's solution.

These facts may account in part for the inconsistency of findings reported in the literature, to show that moist heat of moderate degree causes hydrolysis of certain higher disaccharides and other carbohydrates, such as dextrin and starch.

In carrying out a number of tests preliminary to the major work reported upon in connection with this phase of the investigation, one experiment is worthy of particular consideration,—that made to determine the effect of moist heat upon certain carbohydrates. The temperatures used were those encountered in sterilizing solutions in the autoclave and by the discontinuous method. Three different carbohydrate-containing solutions were made up: (1) distilled water with 0.5 per cent of the carbohydrates to be tested, (2) Dunham's solutions, acidulated by the addition of 0.4 per cent N/1 HCl, separate portions of which contained 1 per cent of the carbohydrates, (3) a plain Dunham's solution which contained 1 per cent of the carbohydrates being tested. Each of these lots was separated in 3 portions. The controls were not heated and were titrated immediately. The second set was autoclaved at 15 pounds (121.3° C.) for 15 minutes, the total length of time above 100° C. being 45 minutes. The third set was exposed in an Arnold steamer for intervals of 20 minutes at about 99.5° C. on 3 consecutive days. The findings appear in table IX, and it will be seen from the tabulated data that sterilization of certain saccharide broths was accomplished by either method without significant hydrolysis of the carbohydrates present when no acid (HCl) was added. Certain of the monosaccharides, as well as the disaccharides lac-

tose and maltose, dissolved in the Dunham's solution appeared to have been slightly altered during the process of sterilization in the Arnold sterilizer, but it seems doubtful if the changes which are indicated by the figures are significant even in the case of lactose and maltose, since, as has been pointed out, these sugars are characterized to a certain extent by a variability in reducing power.

On the other hand, reference to table IX emphasizes clearly the fact that a considerable hydrolysis of the di- and polysaccharides occurred in the acidulated broths exposed in the autoclave, and to a lesser extent in those exposed in the steamer. It also appears that sucrose is most markedly affected. According to my tests, lactose, maltose, and potato starch in slightly acidulated broth are not hydrolyzed by exposure to moist heat in the process of sterilization at 100° C.

The data given below are of interest when compared with the quantitative data presented in table IX. These data, furthermore, serve as a check on the other type of analysis.

TABLE VIII

RESULTS OF COLORIMETRIC TEST WITH IODINE.

Carbohydrate	Controls	Autoclave	Arnold Steamer
Dextrin	Magenta	No color	Very pale magenta
Potato starch	Blue	Magenta	Blue

In the other tables (IX-XIII) are presented data bearing on the quantitative consumption of chosen carbohydrates by a few microorganisms. Nine of the 12 cultures employed were representative strains of the blackleg bacillus and among the 9 were the 4 "species" of this bacillus, which, together with the other strains, were studied comparatively at an earlier date (see Part II). In addition to these, cultures of *Bacillus coli, B. vulgatus,* and a species of yeast (probably a species of *Saccharomyces*) were investigated. For the sake of facilitating comparisons the amounts of carbohydrate consumed, in terms of glucose, are assembled in table XIV.

DISCUSSION

The data presented in tables IX–XIV call for very little further commentary, but the action of strain numbers 195 and 196 (table XI and XII) in the presence of sucrose attract special

attention as being extraordinary for the bacillus represented. It would appear that these strains, unlike others of *Bacillus atrosepticus*, inverted cane sugar faster than the hexose by-products were used up. Also, it will be noted that these strains consumed nearly triple the average amount of sucrose used by the other blackleg strains. In this respect these cultures are comparable with *B. vulgatus*, which is the only other one of the 12 which consumes sucrose so strongly and, in addition, inverts this saccharide faster than it uses up the invert sugar. It is possible that these 2 cultures (Nos. 195 and 196) were contaminated during the course of these experiments. Neither attracted attention because of extraordinary cultural features at the time and so were not plated out.

Figures are presented in tables XI, XIII, and XIV which indicate a very slight consumption of dextrin by the blackleg strains number 187B.1 and 198. Also, it would appear that minute quantities of starch were used by strain No. 201 (table X), and it would seem that lactose was attacked by *B. vulgatus* as well as maltose by the yeast species (tables XIII and XIV). The writer is of the opinion, however, that these data in themselves are slightly misleading. It seems to him more than likely that they appear as the result of small discrepancies which are likely to develop in work of this nature and for reasons which have been stated above. It will be noted that the strain of *Bacillus coli* investigated did not hydrolyze sucrose nor consume it. *Bacillus vulgatus* as cultivated in these experiments did not utilize lactose nor did it use more than about a third as much galactose as glucose and fructose. Both these bacteria, it will be noted, attack with considerable avidity all the other carbohydrates employed. The yeast species used was unable to attack and use carbohydrates presented other than glucose and fructose. These sugars, however, were totally eliminated from the broth by this microorganism.

The several strains of *Bacillus atrosepticus* employed in these tests were quite alike in respect to their attack upon and use of the several carbohydrates presented. It appears likely that 15–20 per cent of the amount of the carbohydrates presented would have been sufficient to supply the strains employed, under the conditions of the experiments. The strains of the blackleg bacillus, furthermore, utilized sucrose, leaving no invert sugar behind except in the cases cited above of strains Nos. 195 and

TABLE IX

EFFECT OF METHOD OF STERILIZATION ON CARBOHYDRATES

Carbohydrates tested	0.5% in aqueous sol.					1% in Dunham's sol. +10 by HC1					1% in Dunham's sol. Not adjusted				
	Contrl.*	Autoclave		Arnold		Contrl.	Autoclave		Arnold		Contrl.	Autoclave		Arnold	
	Total recovery	Total recovery	Gain due to heat	Total recovery	Gain due to heat	Total recovery	Total recovery	Gain due to heat	Total recovery	Gain due to heat	Total recovery	Total recovery	Gain due to heat	Total recovery	Gain due to heat
	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.
Glucose	4.7	4.7	0	4.7	0	10.2	10.2	0	10.2	0	10.6	10.5	0	10.6	0
Fructose	4.1	4.1	0	4.1	0	9.07	9.14	0.07	9.14	0.07	9.1	8.95	0	9.17	0.07
Galactose	4.4	4.1	0	4.1	0	9.26	9.25	0	9.27	0.01	9.15	9.2	0	9.2	0.05
Sucrose	0	0	0	0	0	0	11.5 (11.0)	11.5	11.3 (10.8)	11.3	0	0	0	0	0
Lactose	3.3	2.8	0	3.3	0	6.35	7.27	0.92	6.35	0	5.35	5.44	0.09	5.5	0.2
Maltose	2.32	2.31	0	2.31	0	4.6	6.07	2.67	4.55	0	3.9	3.89	0	4.18	0.28
Dextrin	0	0	0	0	0	0	1.44	1.3	0.85	0.76	0	0	0	0	0
Potato starch	0	0	0	0	0	0	1.04	0.9	0	0	0	0	0	0	0

* Not heated.

TABLE X

CARBOHYDRATE CONSUMPTION OF BACILLUS ATROSEPTICUS STRAINS

	Controls		No. 201				No. 197			
	Total recovery		Total recovery		Consumption		Total recovery		Consumption	
	Carb.*	Gluc.†	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.
	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.
Glucose	9.3	9.3	8.2	8.2	1.1	1.1	8.4	8.4	0.9	0.9
Fructose	8.4	9.2	7.3	8.0	1.1	1.2	7.4	8.0	1.0	1.2
Galactose	8.8	9.8	7.7	8.6	1.1	1.2	7.6	8.5	1.2	1.3
Sucrose	0	9.8 (9.4)‡	0	8.5 (8.2)‡	1.2	1.3	0	8.7 (8.4)‡	1.0	1.1
Lactose	5.8	9.8	4.7	7.1	1.1	1.9	4.7	7.0	1.1	2.0
Maltose	4.8	9.4	4.0	8.6	0.8	0.8	4.1	8.6	0.7	0.8
Dextrin	0	8.1	0	8.1	0	0	0	8.1	0	0
Potato starch	0	8.2	0	8.1	0	0.1	0	9.0	0	0

* Carbohydrate.
† Glucose.
‡ Invert sugar value in parenthesis.

TABLE XI

CARBOHYDRATE CONSUMPTION OF BACILLUS COLI AND B. ATROSEPTICUS STRAINS

	Controls		Bacillus coli				B. atrosepticus							
							No. 187B.1				No. 196			
	Total recovery		Total recovery		Consumption		Total recovery		Consumption		Total recovery		Consumption	
	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.
	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.
Glucose	9.5	9.5	6.0	6.0	3.5	3.5	8.3	8.3	1.2	1.2	7.8	7.8	1.7	1.7
Fructose	8.5	9.2	5.5	6.0	3.0	3.2	7.7	8.3	0.8	0.9	7.5	8.1	1.0	1.1
Galactose	8.7	9.7	5.4	6.0	3.3	3.7	7.8	8.7	0.9	1.0	7.3	8.1	1.4	1.6
Sucrose	0	9.7 (9.3)	0	9.7 (9.3)	0	0	0	8.7 (8.3)	0.9	1.0	2.2* (2.1)	6.0* (5.8)	3.5*	3.7*
Lactose	5.7	9.0	3.3	6.3	2.4	2.7	5.1	7.8	0.6	1.2	4.9	7.8	0.8	1.2
Maltose	4.5	9.4	3.2	6.2	1.3	3.2	4.2	8.7	0.3	0.7	3.2	7.0	1.3	1.4
Dextrin	0	8.2	0	6.8	+	1.4	0	8.0	0	0.2	0	8.2	0	0
Potato starch	0	8.1	0	6.0	+	2.1	0	8.1	0	0	0	8.2	0	0

* These figures seem to indicate an extraordinary sucrose consumption (but see pages 55 and 62). Certain of them indicate that the sucrose was inverted faster than used by the organism. See also No. 195, table XII.

TABLE XII

CARBOHYDRATE CONSUMPTION BY BACILLUS ATROSEPTICUS

	Controls		No. 195				No. 180.2				No. 160.1			
	Total recovery		Total recovery		Consumption		Total recovery		Consumption		Total recovery		Consumption	
	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.
	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.
Glucose	9.5	9.5	8.7	8.7	0.8	0.8	8.2	8.2	1.3	1.3	8.4	8.4	1.1	1.1
Fructose	8.7	9.5	7.6	8.3	1.1	1.2	7.4	8.0	1.3	1.5	7.7	8.4	1.0	1.1
Galactose	8.7	9.7	7.4	8.2	1.3	1.5	7.4	8.2	1.3	1.5	8.2	9.1	0.5	0.6
Sucrose	0	9.7 (9.3)	2.0* (1.9)	6.1 (5.9)	3.4	3.6*	0	7.5 (7.2)	2.1	2.2	0	8.7 (8.4)	0.9	1.0
Lactose	6.0	9.5	5.0	8.0	1.0	1.5	4.9	7.4	1.1	2.1	5.6	7.8	0.4	1.7
Maltose	4.5	9.2	4.4	8.8	0.1	0.4	4.0	8.0	0.5	1.2	3.9	8.6	0.6	0.6
Dextrin	0	8.1	0	8.1	0	0	0	8.1	0	0	0	8.1	0	0
Potato starch	0	8.1	0	8.1	0	0	0		0	0	0	8.1	0	0

* Cf. also No. 196 table XI.

TABLE XIII

CARBOHYDRATE CONSUMPTION BY BACILLUS VULGATUS, A YEAST, AND STRAINS OF B. ATROSEPTICUS

	Controls		Bacillus atrosepticus								Bacillus vulgatus				A yeast			
			No. 198				No. 183.2											
	Total recovery		Total recovery		Consumption		Total recovery		Consumption		Total recovery		Consumption		Total recovery		Consumption	
	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.	Carb.	Gluc.
	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.	mgms.
Glucose	9.4	9.4	8.4	8.4	1.0	1.0	8.2	8.2	1.2	1.2	5.4	5.4	4.0	4.0	0	0	All	All
Fructose	8.6	9.4	7.4	8.0	1.2	1.3	7.2	7.8	1.4	1.6	4.6	5.0	4.0	4.4	0	0	All	All
Galactose	8.7	9.7	7.7	8.5	1.0	1.2	7.8	8.6	0.9	1.1	7.4	8.2	1.3	1.5	9.1	10.0	0	0
Sucrose	0	9.6 (9.2)	0	8.3 (8.0)	1.2	1.3	0	8.2 (7.9)	1.3	1.4	6.0†	7.3 (7.0)	2.2	2.3	0	9.6 (9.2)	0	0
Lactose	5.9	9.0	5.0	7.4	.9	1.6	4.9	6.9	1.0	2.1	5.8	8.9	0.1	0.1	6.0	9.2	0	0
Maltose	4.5	9.3	4.9*	7.4†	?	1.9	4.1	8.5	0.4	.8	2.3	5.9	2.2	3.4	4.5	9.1	0	0.2
Dextrin	0	8.2	0	8.0	0	0.2	0	8.2	0	0	1.3†	4.9	0	3.3	0	8.2	0	0
Potato starch	0	8.1	0	8.2	0	0	0	8.4	0	0	1.0†	4.5	0	3.7	0	8.2	0	0

* Clumps of growth in culture.

† This organism evidently hydrolyzes certain carbohydrates, splitting off invert sugar faster than it consumes it.

TABLE XIV

AMOUNTS OF CARBOHYDRATE CONSUMED BY CERTAIN MICRO-ORGANISMS

Test substance	Mgms. of glucose											
	B. coli	B. vulgatus	A yeast	Bacillus atrosepticus								
				201	198	197	196	195	187B.1	183.2	180.2	160.1
Glucose	3.5	4.0	9.4	1.1	1.1	0.9	1.7	0.8	1.2	1.2	1.3	1.1
Fructose	3.2	4.4	9.4	1.2	1.3	1.2	1.1	1.2	0.9	1.6	1.5	1.1
Galactose	3.7	1.5	0	1.2	1.2	1.3	1.1	1.5	1.0	1.1	1.5	0.6
Sucrose	0	2.3	0	1.3	1.3	1.1	3.7*	3.6*	1.0	1.4	2.2	1.0
Lactose	2.7	0.1	0	1.9	1.6	2.0	1.2	1.5	1.2	2.1	2.1	1.7
Maltose	3.2	3.4	0.2	0.8	1.8	0.8	1.4	0.4	0.7	0.8	1.2	0.6
Dextrin	1.4	3.3	0	0	0.2	0	0	0	0.2	0	0	0
Potato starch	2.1	3.7	0	0.1	0	0	0	0	0	0	0	0

* Probably too high. See pages 55, 62.

196. *Bacillus coli* and *B. vulgatus* were the only organisms investigated that attacked and used dextrin and starch. The latter, however, appears to have been able to break down both of these substances more rapidly than it utilized the derived sugars (table XIII).

In respect to the amounts of carbohydrates actually utilized, it will be noted upon reference to table XIV that with the several microorganisms under observation (excepting the yeast) the amount is relatively small as compared with the total amount supplied (10 mgms. per cc.). The single instance of *Bacillus vulgatus* on fructose is the only one where the actual amount consumed approaches 50 per cent of the total amount of the sugar provided. The different strains of *Bacillus atrosepticus* consumed only about 10 to 20 per cent of the available supply of carbohydrates. *Bacillus coli* utilized about 30 per cent, and *B. vulgatus* about 40 per cent of the supply. In the light of these findings it would appear that the usual recommendation relative to the amount of carbohydrate that should be supplied in a nutrient medium was a most liberal one. *Bacillus coli* and *B. vulgatus* are usually thought of as being particularly active in fermenting certain saccharides, especially when compared to most plant pathogens. As it is possible that there are numerous bacterial species for which the optimum sugar concentration is 1 per cent or even more, it is doubtful if a less liberal recommendation should be made until the whole matter has been more extensively investigated. However, in the light of these findings, as well as those of Besson, Ranque and Senez ('19), Chambers ('20), Wolf and Foster ('21), and others, it appears that *B. coli,* also some plant pathogenic bacteria, require a less amount of sugar than 1 per cent for optimum development. Besides, there is abundant evidence which indicates that most plant pathogenic bacteria require a very much lower sugar concentration than 1 per cent (cf. also sugar consumption by *B. atrosepticus,* table XIV).

Further evidence of the similarity of the several strains of the blackleg bacillus employed throughout these investigations is brought out in tables X–XIII.

General Summary

I

The blackleg disease of Irish potatoes in North America and Europe is caused by a Schizomycete which should bear the name *Bacillus atrosepticus* van Hall.

The following names are to be considered only as synonyms: *Bacillus phytophthorus* Appel, *B. solanisaprus* Harrison, *B. melanogenes* Pethybridge & Murphy.

The index number "5312-32120-2111" very briefly describes *Bacillus atrosepticus* van Hall, the number being based on the results of the writer's comparative studies.[1] A revised description of this organism appears on pages 43–44.

The blackleg parasite grows best at about 26° C. It withstands extremely low temperatures (–28.2°C.) for several hours.

This organism produces acid and a small volume of gas from each of a number of sugars. The gas-producing capacity is relatively weak, but this capacity can be built up to a certain extent by constant cultivation in the presence of the sugars which it is able to utilize.

The pathogen infects the vines and the tubers of the potato.

Virulence of the parasite, as tested by artificial inoculation, appears to be dependent upon a rather delicate balance of temperature and water relations, and upon the sugar content of the tissues inoculated.

The "incubation period" varied in the experiments under observation and appears to be influenced by the same factors mentioned above. No definite "turning point" was observed to occur, as in the case of many animal diseases.

The above conclusions are based on studies of 12 strains of the potato blackleg parasite, including the 4 "species" originally described as being the cause of the disease.

The strains studied were observed to be morphologically similar. That they were very much alike in their cultural characteristics and physiology was abundantly proven by extensive comparative studies (see data on p. 41).

[1]See Descriptive Chart, Society of American Bacteriologists.

II

Quantitative determinations of carbohydrate utilization show that *Bacillus atrosepticus* cannot hydrolyze potato starch or dextrin.

This organism, however, can utilize the saccharides, glucose, fructose, galactose, sucrose, lactose, and maltose.

The several strains (9) of *B. atrosepticus* investigated were similar in respect to their ability to hydrolyze and utilize the saccharides presented.

Bacillus coli can utilize the carbohydrates, lactose, maltose, dextrin, and potato starch, as well as glucose, fructose, and galactose. The strain investigated did not hydrolyze sucrose, nor was the amount of the original of this sugar reduced.

Bacillus vulgatus can consume the saccharides, sucrose, maltose, dextrin, and potato starch, as well as glucose, fructose, and galactose. The strain investigated could not hydrolyze nor consume lactose.

Under the conditions of the experiments a carbohydrate concentration of 0.25 per cent, 0.4 per cent, and 0.5 per cent would have furnished an ample supply for *Bacillus atrosepticus*, *B. coli*, and *B. vulgatus*, respectively.

The yeast species investigated utilized only glucose and fructose, of the carbohydrates presented. Both these saccharides were completely removed by this organism in less than 6 days, under the conditions of the experiment.

The saccharides, sucrose, lactose, maltose, dextrin, and potato starch, were not hydrolyzed appreciably if sterilized in the autoclave when dissolved in the Dunham's solution employed.

The disaccharides, sucrose, lactose, and maltose, were hydrolyzed with about equal rapidity in both the autoclave and the steamer when a very small amount of a mineral acid (0.4 per cent normal HCl) was added to "adjust" the Dunham's solution.

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Explanation of Plate

PLATE 1

Fig. 1. Affected plant showing characteristic blackening of stalk and lower branches.

Fig. 2. Similarly affected plant. Lower parts of main stalk and one of the lateral branches cut away to show destruction and blackening of medullary region.

Fig. 3. Young plants prostrated by the disease. Note external signs at base of stalks. The pathogen moved upward into the stalks from the seed piece.

Fig. 4. Median longitudinal section of a recently infected stem showing disease advancing in the pith.

JENNISON—POTATO BLACKLEG

Explanation of Plate

PLATE 2

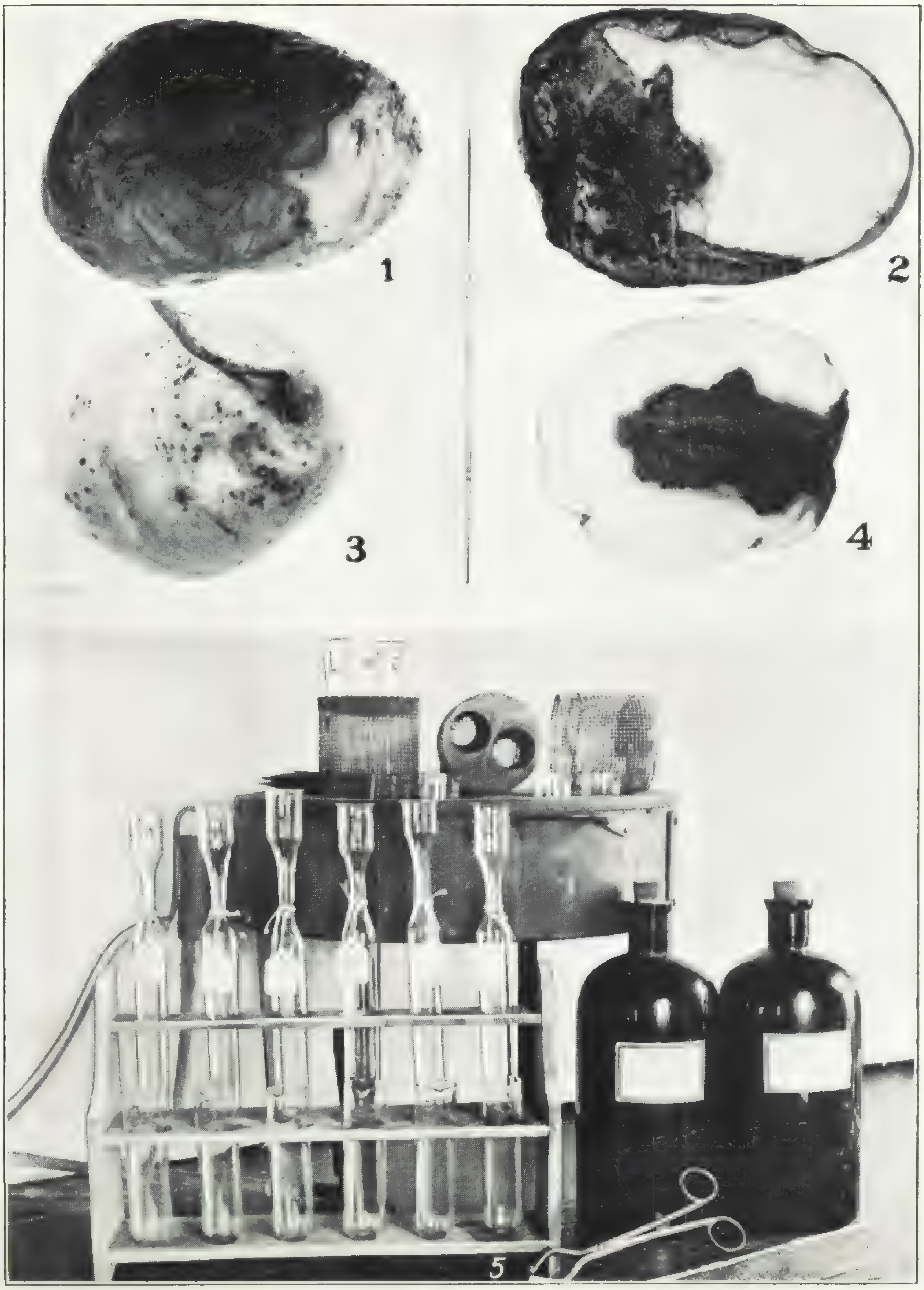
Fig. 1. External view of tuber selected to illustrate characteristics of the rot as well as extent to which it may be manifest externally.

Fig. 2. Internal view of same. The necrosed tissues were quite dry and cheesy.

Fig. 3. External view of affected tuber. The tuber appears to be quite sound except for slight blackening and shrinking of the stolon.

Fig. 4. Internal view of same tuber. Note extent of lesion.

Fig. 5. Apparatus and materials used for determination of sugar consumption.



JENNISON—POTATO BLACKLEG

STUDIES OF SOUTH AMERICAN SENECIOS—I[1]

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The representation of the South American flora in American herbaria has been greatly augmented during the past ten years by several scientific expeditions to South America and by material acquired from botanical correspondents and experienced collectors residing in South America.

Dr. N. L. Britton, Director of the New York Botanical Garden, has generously submitted to the writer for study the extensive suite of specimens of *Senecio,* collected in Colombia by Dr. H. H. Rusby and Dr. F. W. Pennell in 1917 and 1918; and Dr. J. N. Rose, of the United States National Museum, has placed in my hands the excellent series of specimens of this genus obtained by him and his associates on the several expeditions to South America in connection with his studies of the Cactaceae. Dr. B. L. Robinson, Curator of the Gray Herbarium of Harvard University, Mr. William R. Maxon, of the United States National Herbarium, and Dr. Charles F. Millspaugh, of the Field Museum of Natural History, have kindly loaned valuable material of this group. Dr. H. H. Rusby has given valued information and supplied material of certain specimens collected by Miguel Bang. To all these gentlemen the writer is under obligation and takes this opportunity to express his personal gratitude.

A critical study of this relatively large assemblage of specimens of South American Senecios has made it possible to identify many of the earlier species published by Kunth, De Candolle, Weddell, and others, as well as many of the more recent species described by Klatt, Hieronymus, and contemporary authors. It has seemed advisable to record a partial list of the species identified and to present descriptions of those species which appear not to have been previously described.

Senecio aberrans Greenm., sp. nov. Plate 3.

Herba erecta elata; caule stricto striato juventate subarachnoideo denique plus minusve glabrato usque ad inflorescentias folioso; foliis alternis petiolatis oblongo-lanceolatis 10–15 cm. longis 2–4 cm. latis acuminatis acutis leviter sinuato-calloso-serratis basi rotundatis vel cuneatis juventate utrinque arach-

[1] Issued September 28, 1923.

noideo-tomentulosis supra mox glabratis reticulato-nervatis et subinde paulo bullatis, medio nervio venisque subtus prominulis; petiolis 5–7 mm. longis; inflorescentiis terminalibus multicapitatis pyramido-paniculatis, ramis paniculae subracemosis; capitulis heterogamis 12–15 mm. altis radiatis; involucris anguste campanulatis calyculatis; involucri bracteis saepe 13 lineari-lanceolatis 10–12 mm. longis acutis penicillatis glabris vel parce puberulentis, marginibus scariosis; floribus ligulatis plerumque 8, tubo gracile 7 mm. longo, ligula anguste oblonga circiter 7 mm. longa 2 mm. lata 5-nervata flava; floribus disci 12–15 tubulo-campanulatis circiter 1 cm. longis flavibus, limbo basi sensim ampliato aequaliter 5-dentato; pappi setis circiter 1 cm. longis fuscentibus; achaeniis glabris.

Tall erect herb; stem strict, striate, subarachnoid in the early stages, later more or less glabrate, leafy to the inflorescence; leaves alternate, petiolate, oblong-lanceolate, 10–15 cm. long, 2–4 cm. broad, acuminate, acute, shallowly sinuate-callous-serrate, rotund or cuneate at the base, arachnoid-tomentulose on both surfaces in the early stages, soon glabrate, reticulately nerved, and somewhat bullate above, the median nerve and lateral veins conspicuous beneath; petioles 5–7 mm. long; inflorescence a terminal many-headed pyramidal panicle; heads heterogamous, 12–15 mm. high, radiate; involucres narrowly campanulate, calyculate; bracts of the involucre usually 13, linear-lanceolate, 10–12 mm. long, acute, penicillate, glabrous or sparingly puberulent, margins scarious; ligulate flowers usually 8, tube slender, 7 mm. long, ligule narrowly oblong, about 7 mm. long, 2 mm. broad, 5-nerved, yellow; flowers of the disk 12–15, tubular-campanulate, about 1 cm. long, yellow, the limb gradually ampliated from the base, equally 5-dentate; setae of the pappus about 1 cm. long, tawny; achenes glabrous.

Colombia: depression in prairie, Mariquita, Department of Tolima, alt. 250–300 m., January 7, 1918, *Pennell 3636* (N. Y. Bot. Gard. Herb., Mo. Bot. Gard. Herb., and U. S. Nat. Herb.), TYPE; Honda, August, 1919, *Bro. Ariste-Joseph A 356* (U. S. Nat. Herb.); without definite locality, coll. of 1917, *Rusby & Pennell* (N. Y. Bot. Gard. Herb.).

This species is somewhat aberrant in the genus *Senecio* on account of the relatively long and slightly conical-tipped or obtusish style-branches. In general, however, it conforms with the technical characters of the genus.

Senecio abietinus Willd. ex Wedd. Chlor. And. 1:100. 1855-57.

Colombia: Guadalupe, near Bogotá, September, 1907, *Bro. Ariste-Joseph A28* (U. S. Nat. Herb.); Verjón, September, 1917, *Bro. Ariste-Joseph A154* (U. S. Nat. Herb. and Mo. Bot. Gard. Herb.); without definite locality or date of collection, *Bro. Ariste-Joseph* (Gray Herb., Mo. Bot. Gard. Herb., and U. S. Nat. Herb. No. 888284); bushy slope, above Bogotá, Department of Cundinamarca, alt. 2200–2800 m., August 16, 1917, *Rusby & Pennell 1289* (N. Y. Bot. Gard. Herb. and Field Mus. Herb.); open rocky mountain slope, Chapinero, near Bogotá, Department of Cundinamarca, alt. 3000–3100 m., September 18–23, 1917, *Pennell 2019* (N. Y. Bot. Gard. Herb., Mo. Bot. Gard. Herb., and U. S. Nat. Herb.); Páramo de Choachi, near Bogotá, alt. 3700 m., August 8, 1922, *Killip & Bro. Ariste-Joseph 11928* (U. S. Nat. Herb.); Cundinamarca, coll. of 1883, *Lehmann 2406* (Gray Herb.); coll. of 1861, ex Herb. *J. de Parsaval-Grandmaison 92* (Gray Herb.).

Venezuela: Páramo de Tama, head of Tachira River, alt. 2440 m., March, 1911, *Wilfred H. Osgood* (Field Mus. Nat. Hist. Herb.).

Senecio adenophyllus Meyen & Walp. in Nov. Act. Nat. Cur. **19**: Suppl. 1, p. 282. 1843; Walp. Rep. **6**:271. 1846-47; Wedd. Chlor. And. **1**:112. 1855-57.

Bolivia: vicinity of Oruro, August 18, 1914, *Mr. & Mrs. J. N. Rose 18932* (U. S. Nat. Herb. and Mo. Bot. Gard. Herb.).

Peru: below Pampa del Arrieros, August 23, 1914, *Mr. & Mrs. J. N. Rose 18951* (U. S. Nat. Herb. and Mo. Bot. Gard. Herb.).

The leaves of this species are apparently quite variable in size and outline. Walper's description reads "foliis sessilibus confertis ovato-lanceolatis acuminatis grosse serratis vel subintegris lineari-lanceolatis." In the Gray Herbarium there is a drawing of Meyen's original material from Peru which shows a narrowly lanceolate, coarsely and irregularly dentate form of leaf. Although the leaves on the specimens above cited are narrower than they would appear to be in the type, yet the specimens collected by Mr. and Mrs. Rose in all probability represent a narrow-leaved form of the above species.

Senecio adenotrichus DC. Prodr. **6**:416. 1837.

Chile: Quillota, *Bertero 1044* (Mo. Bot. Gard. Herb.); without locality, *Styles* (Phil. Acad. Nat. Sci. Herb.); Palos Quernados, October 4, 1914, *Mr. & Mrs. J. N. Rose 19187* (U. S. Nat. Herb.

and Mo. Bot. Gard. Herb.); vicinity of Choapa, and vicinity of Illapel, October 6. 1914, *Mr. & Mrs. J. N. Rose 19193, 19362, 19237* (U. S. Nat. Herb.); San Felipe, September 25, 1919, *Holway & Holway 67* (Gray Herb.).

Senecio alternifolius Greenm., comb. nov.

Gynoxis alternifolia Schz. Bip. in Bull. Soc. Bot. Fr. **12**:80. 1865, name only; in Linnaea **34**:531. 1866, name only; Rusby in Mem. Torr. Bot. Club **6**:67. 1896, description.

Bolivia: vicinity of Sorata, alt. 3000–3200 m., May, 1859, *Mandon 131* (Gray Herb.); vicinity of Mapiri, alt. 2435 m., September, 1892, *Bang 1574* (Mo. Bot. Gard. Herb.); Unduavi, September, 1894, *Bang 2477* (Mo. Bot. Gard. Herb. and Gray Herb.), distributed as *"Eupatorium trichotomum* Sch. Bip."; Unduavi, Noryungas, alt. 3300 m., November, 1910, *Buchtien 3078* (N. Y. Bot. Gard. Herb.).

The two morphological characters, namely, opposite leaves and conical tipped style-branches, ordinarily relied upon to distinguish *Gynoxis* from *Senecio,* are at best very unsatisfactory. The latter character does not hold, since the same type of style-branch occurs in a number of species which are accepted unqualifiedly as true Senecios. The one character separating these two genera is the leaf arrangement. In only one case, among the several collections cited above, namely, Bang's No. 1574, do the leaves approach an opposite position on the stem, and even here they are only subopposite. In all other regards the species in question is a typical *Senecio,* hence it is transferred to that genus.

Senecio andicola Turcz. Bull. Soc. Nat. Mosc. **24**^{2}:91. 1851.

S. vernicosus Schz. Bip. var. *major* Wedd. Chlor. And. **1**:94. 1855-1857.

S. ledifolius Schz. Bip. acc. to Wedd. *l. c.*

Colombia: moist open places, Páramo de Ruiz, Department of Tolima, alt. 3400–3700 m., December 16-17, 1917, *Pennell 3076* (N. Y. Bot. Gard. Herb., U. S. Nat. Herb., and Mo. Bot. Gard. Herb.).

A study of additional material may show that this species should be merged with *S. ledifolius* (HBK.) DC., but at present it seems desirable to regard it as specifically distinct, on account of the larger leaves and broader calyculate bracteoles of the involucre.

Senecio apiculatus Schz. Bip. ex Wedd. Chlor. And. **1**:128. 1855-57.

Venezuela: Páramo de Timotes, State of Táchira, alt. 3000–3500 m., March, 1910, *Jahn 6* (U. S. Nat. Herb.); Páramo Piedras Blancas, alt. 4000 m., March 27, 1915, *Jahn 426* (U. S. Nat. Herb.); Páramo de La Sal, Mérida, alt. 3400 m., September, 1921, *Jahn 556* (U. S. Nat. Herb.); Páramo de Timotes, Mérida, alt. 3600 m., January 22, 1922, *Jahn 789* (U. S. Nat. Herb.).

This species is known in Venezuela under the common name of "Romerito cenizo."

Senecio arboreus Greenm., comb. nov.

Cacalia arborea HBK. Nov. Gen. & Sp. 4:163, *pl. 359.* 1820.

Colombia: shrub zone, below Páramo del Chaquiro, Cordillera Occidental, Department of Bolivar, alt. 2800–3100 m., February 24, 1918, *Pennell 4313* (N. Y. Bot. Gard. Herb.).

Senecio aridus Greenm., sp. nov. Plate 4.

Herbaceus perennis ubique floccoso-tomentosus; caule erecto vel suberecto circiter 3 dm. alto plus minusve glabrato; foliis alternis inferioribus oblanceolatis 3.5–8 cm. longis 4–10 mm. latis acutis vel obtusis calloso-denticulatis vel integris marginibus revolutis juventate utrinque floccoso-tomentosis supra plus minusve glabratis, eis caulinis lanceolatis sessilibus et brevi-decurrentibus; inflorescentiis terminalibus paucicapitatis magnis radiatis; involucris campanulatis calyculatis; involucri bracteis 21 lineari-lanceolatis circiter 13 mm. longis 2–3 mm. latis parce tomentulosis ad apicem penicillatis; floribus liguliferis 15-20, ligulis late oblongis 13–15 mm. longis 5–7 mm. latis pallide flavibus; floribus disci circiter 125; achaeniis glabris.

Herbaceous perennial, floccose-tomentulose throughout; stem erect or suberect, about 3 dm. high, more or less glabrate; leaves alternate, the lowermost oblanceolate, 3.5–8 cm. long, 4–10 mm. broad, acute or obtuse, callous-denticulate, or entire, revolute-margined, in the early stages floccose-tomentose on both surfaces, more or less glabrate above; upper stem-leaves lanceolate, sessile and short-decurrent; inflorescence terminal, few-headed; heads large, radiate; involucre campanulate, calyculate; bracts of involucre 21, linear-lanceolate, about 13 mm. long, 2–3 mm. broad, sparingly tomentulose, penicillate at the apex; ray-flowers 15–20, rays broadly oblong, 13–15 mm. long, 5–7 mm. broad, light yellow; disk-flowers about 125; achenes glabrous.

Colombia: dry open places, Páramo de Ruiz, Department of Tolimo, alt. 3800–4300 m., December 16-17, 1917, *Pennell 3037* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.), TYPE.

This species is related to *S. latiflorus* Wedd., but differs in being floccose-tomentulose throughout, in having much smaller and shorter leaves, and in having smaller heads with fewer flowers.

Senecio auritus Wawra Itin. Princ. S. Coburg. 2:47. 1888.

Brazil: vicinity of Itatiaya, July 26-30, 1915, *Rose & Russell 20559* (U. S. Nat. Herb., fragment and photograph in Mo. Bot. Gard. Herb.).

Senecio brachycodon Baker in Mart. Fl. Bras. 6^{3}:319. 1884.

Brazil: vicinity of Itatiaya, July 26-30, 1915, *Rose & Russell 20506* (U. S. Nat. Herb., fragment and photograph in Mo. Bot. Gard. Herb.).

Senecio Buchtienii Greenm., sp. nov.

Frutex 2–3 m. altus; ramis ramulisque striato-angulatis persistenter papilloso-hispidis; foliis alternis petiolatis lanceolatis vel lanceolato-oblongis 5–8 cm. longis 7–20 mm. latis sinuato-dentatis acutis basi cuneatis juventate parce pubescentibus denique supra glabratis et atro-viridibus subtus pallidioribus nisi in nervo medio glabratis; petiolis usque ad 1.5 cm. longis; inflorescentiis terminalibus vel lateralibus laxe corymboso-cymosis bracteatis fusco-pubescentibus, bracteis lanceolatis vel linearibus; capitulis heterogamis 1–1.5 cm. altis; involucris anguste campanulatis calyculatis, squamellis calyculatis linearibus 3 mm. vel minus longis; involucri bracteis 8 lineari-lanceolatis 8–9 mm. longis glabris; floribus liguliferis 5, ligulis parvis linearibus 5–6 mm. longis plerumque bidentatis flavibus; floribus disci 10–12; pappi setis albidis ad corollam subaequantibus; achaeniis glabris.

Shrub 2–3 m. high; branches and branchlets striate-angled, persistently papillose-hispid; leaves alternate, petiolate, lanceolate or lanceolate-oblong, 5–8 cm. long, 7–20 mm. broad, sinuate-dentate, acute, cuneate at the base, in the young stages sparingly pubescent, later becoming glabrous and dark green above, paler beneath and glabrous or hirsute pubescent on the midrib; petioles 1.5 cm. or less in length; inflorescence terminating the stem and lateral branches in bracteate tawny pubescent loose corymbose cymes; bracts of the inflorescence lanceolate to linear; heads heterogamous, 1–1.5 cm. high; involucre narrowly campanulate, calyculate, bracteoles linear, 3 mm. or less in length; bracts of the involucre 8, linear-lanceolate, 8–9 mm. long, glabrous; ligulate flowers 5, ligules small, linear, 5–6 mm. long,

usually bidentate, yellow; flowers of the disk 10–12; pappus white, about as long as the corolla; achenes glabrous.

Bolivia: Unduavi, Noryungas, alt. 3300 m., November, 1910, *O. Buchtien 3087* (U. S. Nat. Herb., N. Y. Bot. Gard. Herb., fragment and photograph in Mo. Bot. Gard. Herb.), TYPE.

This species appears to be most nearly related to *S. Sepium* Schz. Bip. ex Rusby in Bull. N. Y. Bot. Gard. 4:394. 1907, but differs in having lanceolate leaves, which are cuneate at the base, instead of ovate-cordate leaves, and in having persistently papillose-hispid stems.

Senecio chaquiroensis Greenm., sp. nov.

Frutex scandens; ramis ramulisque teretibus dense fulvo-hispidis; foliis alternis petiolatis elliptico-oblongis 2.5–5 cm. longis 1.2–2 cm. latis mucronatis vel obtusis calloso-denticulatis vel subintegris supra glabris subtus pallidioribus et valde reticulato-venosis plus minusve puberulentis, marginibus revolutis; petiolis 5–7 mm. longis canaliculatis puberulentis vel glabris; inflorescentiis paniculatis fulvo-hispidis; capitulis homogamis; involucris brevi-calyculatis anguste campanulatis, bracteolis calyculatis triangulari-lanceolatis 1–2 mm. longis acutis ciliatis; bracteis involucri 8 anguste oblongis vel lineari-lanceolatis circiter 5 mm. longis extrinsecus glabris acutis penicillatisque; floribus 12; corollis 3–4 mm. longis glabris luteo-albidis pappi setis albidis subaequantibus; achaeniis glabris.

Stem scandent, ligneous; branches and branchlets terete, densely tawny hispid; leaves alternate, petiolate, elliptic-oblong, 2.5–5 cm. long, 1.2–2 cm. broad, mucronate or obtuse, callous-denticulate or subentire, glabrous above, paler and strongly reticulately veined and more or less puberulent beneath, margins revolute; petioles 5–7 mm. long, canaliculate, puberulent or glabrous; inflorescence paniculate, tawny hispid; heads homogamous; involucres short-calyculate, narrowly campanulate, the calyculate bracteoles triangular-lanceolate, 1–2 mm. long, acute, ciliate; bracts of the involucre 8, narrowly oblong or linear-lanceolate, about 5 mm. long, glabrous on the outer surface, acute and penicillate; flowers 12; corollas 3–4 mm. long, glabrous, about as long as the white setae of the pappus; achenes glabrous.

Colombia: shrub zone, below Páramo de Chaquiro, Cordillera Occidental, Department of Bolivar, alt. 2800–3100 m., February 24, 1918, *Pennell 4290* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.).

This species is related to *S. theaefolius* Benth., but differs in having the branches and inflorescence distinctly tawny and densely hispid pubescent, in having the lower leaf-surface more or less puberulent, especially on the midrib, lateral nerves and reticulated veins, and in the somewhat shorter involucre and smaller flowers.

Senecio Chinogeton Wedd. forma **macrocephalus** Hieron. in Engl. Bot. Jahrb. **19**:64. 1894.

Colombia: in bushy mountain valley, Rio San Cristobal, near Bogotá, Department of Cundinamarca, alt. 3000–3300 m., September 20-26, 1917, *Pennell 2047* (N. Y. Bot. Gard. Herb., Mo. Bot. Gard. Herb., and U. S. Nat. Herb.).

The specimens under Pennell's No. 2047 show only the complete inflorescence with mature, discoid, nodding heads, and a few of the uppermost stem-leaves. From the original description of the above species they would seem to be conspecific and probably represent the form "*macrocephala*", briefly characterized by Professor Hieronymus.

Senecio clavifolius Rusby in Mem. Torr. Bot. Club **3**:64. 1893.

S. attenuatus var. *microphyllus* Britt. in Bull. Torr. Bot. Club **19**:264. 1892.

Bolivia: Talca Chugiaguillo, April, 1890, *Bang 792* (U. S. Nat. Herb., Phil. Acad. Nat. Sci. Herb., Field Mus. Nat. Hist. Herb., and Mo. Bot. Gard. Herb.); near La Paz, alt. 3500 m., October, 1885, *Rusby 1691* (U. S. Nat. Herb., Phil. Acad. Nat. Sci. Herb., and Field Mus. Nat. Hist. Herb.).

Senecio comosus Schz. Bip. in Bonplandia **4**:52, 55. 1856, nomen subnudum; Bull. Soc. Bot. Fr. **12**:180. 1865, name only; Linnaea **34**:531. 1866, name only; Wedd. Chlor. And. **1**:129. 1855-57, description.

Bolivia: coll. of 1856-61, *Mandon 136* (Gray Herb.); vicinity of Cochabamba, coll. of 1891, *Bang 1240* (Mo. Bot. Gard. Herb., Phil. Acad. Nat. Sci. Herb., and Gray Herb.); Cochabamba, April 13-21, 1892, *Otto Kuntze* (U. S. Nat. Herb., No. 702117).

Mr. Bang's No. 1240 was taken to represent *Senecio culcitioides* Schz. Bip., which it resembles habitally, and distributed as that species. The specific name, however, was inadvertently written on the label as "*calcitoides,*" and the plant was referred to in Mem. Torr. Bot. Club **6**:65. 1896 as "*Senecio culcitroides* Wedd." The heads of the Bang plant are distinctly radiate, not discoid as in *L. culcitioides* Schz. Bip. The discoid heads and the very broad, conspicuous, almost glabrous midvein on the lower

side of the leaf of *S. culcitioides* serve to separate it from *S. comosus.*

Senecio culcitioides Schz. Bip. in Bonplandia 4:55. 1856, name only and by error as *"cultitoides"*; in Lechler, Berb. Am. Austr. 57. 1857, name only; in Wedd. Chlor. And. 1:103. 1855-57, description; in Bull. Soc. Bot. Fr. 12:80. 1865, name only; in Linnaea 34:530. 1866, name only.

Colombia: dry open places, Páramo de Ruiz, Department of Tolima, alt. 3500–4100 m., December 16-17, 1917, *Pennell 3026* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.).

Bolivia: *Mandon 116* (Gray Herb.).

Dr. Pennell's specimen here cited agrees in all essential details with the original description of the above species except in the number of involucral bracts which, according to Weddell, is 12–15. In the specimen at hand the involucre consists definitely of 21 bracts, disposed in a single series. The subtending bracteoles are relatively conspicuous, yet they do not form a part of the true uniseriate involucre; and on this account the plant in question cannot be referred properly to *Culcitium.* In habit and foliar characters Dr. Pennell's specimen simulates *Culcitium longifolium* Turcz., but that species is described as having a multiseriate involucre.

Senecio decompositus Schz. Bip. ex Hieron. in Engl. Bot. Jahrb. 28:634. 1901.

Colombia: Santa Marta Expedition, 1898-9, *Herbert H. Smith 1989* (Mo. Bot. Gard. Herb., N. Y. Bot. Gard. Herb., and Field Mus. Nat. Hist. Herb.).

The specimens here cited agree in all details with the original description of the above species by Professor Hieronymus. The rather leafy panicle in the specimens at hand varies from 2 to 3 dm. in length and from 1 to 2.5 dm. in breadth. The heads are very numerous, relatively small, and bear regularly, as far as examined, 10 flowers. The collector's comments on the specimens collected read,—"A loosely twining vine, to 20 feet. Moderately common on the Sierra del Libano thickets or dry forest on ridges, 6000–7000 feet. Begins to flower about Jan. 15. The specimens were collected Jan. 25, at 6000 feet. Flowers rich yellow; leaves polished. This is one of the most showy species of the order; a plant in full bloom is a fine sight."

Senecio ericaefolius Benth. Pl. Hartw. 208. 1845.

Peru: western Cordillera, opposite Huancabamba, alt. 2400–

2900 m., September 26, 1911, *C. H. T. Townsend A207* (Field Mus. Nat. Hist. Herb.).

Senecio formosus HBK. Nov. Gen. & Sp. 4:177. 1820; DC. Prodr. **6**:428. 1837; Wedd. Chlor. And. **1**:125. 1855-57, excl. γ. *subtruncatus.*

S. Tabacon Turcz. in Bull. Soc. Nat. Mosc. 24^2:91. 1851.

Colombia: *Moritz 1400* (Gray Herb.); Páramo de Buena Vista, Huila group, Central Cordillera, State of Cauca, alt. 3000–3600 m., January, 1906, *Jahn 1117* (U. S. Nat. Herb.); moist bushy valley, Páramo de Choachi, near Bogotá, Department of Cundinamarca, alt. 3000–3100 m., September 27, 1917, *Pennell 2237* (N. Y. Bot. Gard. Herb.); in swale, "Rosalito", near Páramo de Ruiz, Department of Tolima, alt 2800–3100 m., December 15-17, 1917, *Pennell 2943* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.); Laguna de Buitrago, alt. 3000 m., December, 1919, *Bro. Ariste-Joseph A 503, A 504, A 505* (U. S. Nat. Herb.); Laguna Coche, May 3, 1876, *Andre 3092* (N. Y. Bot. Gard. Herb.).

Venezuela: Páramo de la Cristalina, State of Trujillo, alt. 2900 m., December 20, 1910, *Jahn 13* (U. S. Nat. Herb.).

When *Senecio formosus* HBK. was published originally it was doubtfully attributed to "Nova Hispania," or Mexico. Weddell pointed out that the species is a South American one, and that its erroneous assignment to Mexico was probably due to a confusion of labels accompanying the original material. Weddell's treatment of the species, however, would indicate that he regarded it as a polymorphic species, since he included *S. Tabacon* Turcz. as a synonym and then characterized three varieties, namely, *angustifolius*, *latifolius*, and *subruncinatus*. To the writer the last-named variety seems to merit specific rank and is so treated in this paper. The specimens here cited as representing *S. formosus* show considerable variation in leaf-outline and in the degree of pubescence on the achenes. In Pennell's No. 2943 the achenes are but slightly hirtellous, while in all the others the hirtellous character is very distinct. The species is well marked by the glandular-villous hairs on stem, leaves, and involucre, and by the purple rays and numerous disk-flowers.

Senecio genuflexus Greenm., sp. nov.

Frutex scandens; caulibus vel ramis gracilibus glabris plus minusve flexuosis vel geniculatis; foliis alternis petiolatis oblongo-lanceolatis vel ovato-lanceolatis 2.5–6 cm. longis 1–2.5 cm. latis acutis vel acuminatis submucronatisque integris vel subin-

tegris basi rotundatis vel cuneatis supra glabris subtus juventate parce arachnoideo-puberulentis mox glabratis; petiolis .5–2 cm. longis paululo alatis et basi abrupte ampliatis auriculato-amplexicaulibusque; inflorescentiis terminalibus multicapitatis paniculatis; rhachis paniculatae geniculatis glabris vel parcissime puberulentis; capitulis discoideis 6–8 mm. altis; involucris anguste campanulatis calyculatis; bracteis involucri saepe 8 lineari-lanceolatis 3–3.5 mm. longis glabris; floribus disci 12–14; pappi setis albidis; achaeniis glabris.

Scandent shrub; stems slender, glabrous, more or less flexuous or geniculate; leaves alternate, petiolate, oblong-lanceolate to ovate-lanceolate, 2.5–6 cm. long, 1–2.5 cm. broad, acute or acuminate and submucronate, entire or subentire, rotund or cuneate at the base, glabrous above, in the early stages very sparingly arachnoid-puberulent beneath but soon glabrate; petioles .5–2 cm. long, narrowly winged, abruptly expanded at the base and auriculate-amplexicaul; inflorescence a terminal many-headed panicle; rhachis of the panicle geniculate, glabrous or very sparingly puberulent; heads discoid, 6–8 mm. high; involucre narrowly campanulate, calyculate; bracts of the involucre usually 8, linear-lanceolate, 3–3.5 mm. long, acute, glabrous; flowers of the disk 12–14; bristles of the pappus white; achenes glabrous.

Colombia: thickets on ridges, Sierra del Libano, Santa Marta, alt. 1800–2000 m., January 19, 1899, *Herbert H. Smith 1987* (Mo. Bot. Gard. Herb., TYPE; Gray Herb., in part; U. S. Nat. Herb., in part; N. Y. Bot. Gard. Herb., in part; Field Mus. Nat. Hist. Herb.).

The plant here cited was distributed under the name "*Senecio decompositus* Schz. Bip." with which species it was doubtfully identified, but from which it is readily distinguished by the stipule-like amplexicaul appendages at the base of the petiole and by the strongly geniculate main axis of the inflorescence.

Senecio involucratus (HBK.) DC. Prodr. **6**:422. 1837; Wedd. Chlor. And. **1**:93. 1855-57.

Cacalia involucrata HBK. Nov. Gen. & Sp. **4**:166. 1820.

Aetheolaena involucrata Cass. Dict. Sci. Nat. 48:453. 1827.

Ecuador: Nabón, September 25, 1918, *Rose, Pachano & Rose 22995* (U. S. Nat. Herb. and N. Y. Bot. Gard. Herb.).

Senecio iodopappus Schz. Bip. in Bonplandia 4:55. 1856, name only (*jodopappus*); Wedd. Chlor. And. **1**:116, *pl. 20, fig. B.* 1855-57.

Bolivia: Lake Titicaca, May, 1910, *Buchtien 3106* (U. S. Nat. Herb.); vicinity of Oruro, August 18, 1914, *Mr. & Mrs. J. N. Rose 18926* (U. S. Nat. Herb.).

Senecio laciniatus HBK. Nov. Gen. & Sp. 4:175. 1820.

S. pimpinellifolius var. *laciniata* Hieron. in Engl. Bot. Jahrb. 28:634. 1901.

Ecuador: vicinity of Zaragura, September 28, 1918, *Rose, Pachano & Rose 23143* (U. S. Nat. Herb.).

Peru: *Mathews 87* (Gray Herb.).

Senecio latiflorus Wedd. Chlor. And. 1:125. 1855-57.

Colombia: dry, open, rocky places, Páramo de Ruiz, Department of Tolima, alt. 3700–4200 m., December 16, 17, 1917, *Pennell 3033* (N. Y. Bot. Gard. Herb., U. S. Nat. Herb., Mo. Bot. Gard. Herb., and Gray Herb.); in moist lands, Ruiz, alt. 3000–3500 m., coll. of 1918, *Dawe 749*, and *763* (N. Y. Bot. Gard. Herb.); *Triana 98* (Gray Herb.).

Senecio lophophilus Greenm., sp. nov. Plate 5.

Fruticulus .5–1 m. altus plus minusve diffusus ubique glabrus; caulibus teretibus striatis brunneis; foliis alternis petiolatis lanceolatis 3–6 cm. longis 1–2 cm. latis acutis sinuato-dentatis basi cuneatis utrinque glabris luteo-viridibus; petiolis usque ad 5 mm. longis; inflorescentiis terminalibus dense paniculatis 7 cm. longis 5 cm. latis; capitulis brevi-pedunculatis parvis 6–7 mm. altis discoideis; involucris anguste campanulatis parce calyculatis; bracteis involucri 8 lineari-lanceolatis circiter 5 mm. longis acutis glabris stramineis; floribus 10–12, corollis 5–6 mm. longis flavibus; achaeniis glabris.

Small shrub, .5–1 m. high, more or less diffuse, glabrous throughout; stems terete, striate, brownish; leaves alternate, petiolate, lanceolate, 3–6 cm. long, 1–2 cm. broad, acute, sinuate-dentate, cuneate at the base, glabrous on both surfaces, yellowish green; petioles 5 mm. or less in length; inflorescence a dense terminal panicle, 7 cm. long, 5 cm. broad; heads short-pedunculate, small, 6–7 mm. high, discoid; involucre narrowly campanulate, sparingly calyculate; bracts of the involucre 8, linear-lanceolate, about 5 mm. long, acute, glabrous, stramineous; flowers, 10–12, corollas 5–6 mm. long, yellow; achenes glabrous.

Colombia: open lands on San Lorenzo Ridge, Santa Marta, alt. 2000–2300 m., January 27, 1899, *Herbert H. Smith 1988* (N. Y. Bot. Gard. Herb., fragment and photograph in Mo. Bot. Gard. Herb.).

In general the species here described stands near *S. prunifolius* Wedd., but it differs in leaf-outline and in being glabrous throughout.

Senecio Magnusii Hieron. in Engl. Bot. Jahrb. 28:642. 1901.

Colombia: edge of tree "island" in páramo valley, Mt. Chuscal, west of Zipaquira, Department of Cundinamarca, alt. 3200–3300 m., October 22, 1917, *Pennell 2589* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.); forest on mountain, south of Sibate, Department of Cundinamarca, alt. 2800–3000 m., October 13-15, 1917, *Pennell 2481* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.).

The specimens here cited agree in all essential details with the original description of *S. Magnusii* Hieron. and are hereto referred pending a comparison with type material of this species. The specimens at hand also possess several characters in common with *L. disciformis* Hieron.

Senecio medullosus Schz. Bip. in Bull. Soc. Bot. Fr. **12**:80. 1865, name only; in Linnaea **34**:531. 1866, name only.

Verisimiliter frutex erectus; caulibus ramisque teretibus vel plus minusve striato-angulatis glabris; foliis alternis petiolatis lanceolatis vel lanceolato-oblongis 5–18 cm. longis 1–4.5 cm. latis acuminatis acutis remote calloso-denticulatis vel sinuato-subserratis basi cuneatis juventate parcissime puberulentis nisi in nervo supra medio utrinque mox glabratis; petiolis circiter 1 cm. longis; inflorescentiis terminalibus paniculatis patentibus parce puberulentis; capitulis heterogamis 10–15 mm. altis; involucris campanulatis, brevi-calyculatis; bracteis involucri 13, lineari-lanceolatis vel lanceolato-oblongis 7–8 mm. longis 1.5–2 mm. latis acutis vel obtusis glabris viridibus vel plus minusve purpurascentibus; floribus liguliferis 6–8 purpurascentibus, tubo gracile 5–10 mm. longo curvato, ligulis parvulis oblongis circiter 5 mm. longis; floribus disci 18–20, corollis tubulo-campanulatis aequaliter 5-dentatis plus minusve purpurascentibus; pappi setis albidis corollo brevioribus; achaeniis striatis glabris.

Shrub, probably erect; stem and branches terete or more or less striate-angled, glabrous; leaves alternate, petiolate, lanceolate or lanceolate-oblong, 5–18 cm. long, 1–4.5 cm. broad, acuminate, acute, remotely callous-denticulate or sinuate-subserrate, cuneate at the base, glabrous or very sparingly puberulent in the young stages, soon becoming glabrous on both surfaces except along the midrib above; petioles about 1 cm. long; inflorescence a terminal spreading sparingly puberulent panicle; heads heter-

ogamous, 10–15 mm. high; involucres campanulate, short-calyculate; bracts of the involucre 13, linear-lanceolate or lanceolate-oblong, 7–8 mm. long, 1.5–2 mm. broad, acute or obtuse, glabrous except at the penicillate tip, green or more or less purple; ligulate flowers 6–8, purple, tube slender, 5–10 mm. long, somewhat outwardly curved, ligules small, oblong, about 5 mm. long; disk-flowers 18–20, corollas tubular-campanulate, equally 5-dentate, more or less purple; pappus bristles white, shorter than the corollas; achenes striate, glabrous.

Bolivia: vicinity of Sorata, alt. 2900 m., October, 1858, *Mandon 147* (Gray Herb., fragment and photograph in Mo. Bot. Gard. Herb.), TYPE; Unduavi, Noryungas, alt. 3300 m., November, 1910, *Buchtien 3086* (N. Y. Bot. Gard, Herb., fragment and photograph in Mo. Bot. Gard. Herb.); San Felipe, Provincia de Sur Yungas, March 20, 1920, *Holway & Holway 627* (U. S. Nat. Herb.).

The species here described bears a superficial resemblance to *S. biacuminatus* Rusby, but differs in having purple flowers and in having a long slender tube and very small rays to the ray-flowers. It is also related apparently to *S. reflexus* HBK., which is said to have very entire reflexed leaves.

Senecio Microchaete (Benth.) Wedd. Chlor. And. 1:100. 1855-57.

Microchaete corymbosa Benth. Pl. Hartw. 196. 1845.

Colombia: near Bogotá, *Hartweg 1086* (Gray Herb.); wet páramo valley, Mt. Chuscal, west of Zipaquira, Department of Cundinamarca, alt. 3100–3300 m., October 22, 1917, *Pennell 2580* (N. Y. Bot. Gard. Herb., Field Mus. Nat. Hist. Herb., U. S. Nat. Herb., and Mo. Bot. Gard. Herb.); Guadalupe, near Bogotá, July 10, 1917, *Bro. Ariste-Joseph A14* (U. S. Nat. Herb.).

Doctor Pennell's specimens agree well with Weddell's characterization of the above species and also with the original description and specimen of *Microchaete corymbosa* Benth. on which *Senecio Microchaete* Wedd. was founded. The material also accords in all essential details with the description of *Senecio pungens* (HBK.) DC., from which it is doubtful if *S. Microchaete* Wedd. can be separated satisfactorily. It seems desirable, however, to retain Weddell's name until a careful comparison can be made with the type or authentic specimens of *L. pungens* (HBK.) DC.

Senecio modestus Wedd. Chlor. And. 1:105, *pl. 18B.* 1855-1857, not Phil. in Linnaea 28:745. 1856.

Bolivia: without locality, *Bang 1890* (Mo. Bot. Gard. Herb., Phil. Acad. Nat. Sci. Herb., Gray Herb., and U. S. Nat. Herb.).

The specimen here cited agrees in all essential characters with the original description and illustration of the above species. Mr. Bang's No. 1890 was referred doubtfully by Doctor Rusby, Bull. N. Y. Bot. Gard. 4:397. 1907, to *S. rhizomatus* Rusby from which it is amply distinct.

Senecio nubigenus HBK. Nov. Gen. & Sp. 4:174. 1820.

S. pimpinellifolius var. *nubigena* (HBK.) Hieron. in Engl. Bot. Jahrb. 28:634. 1901.

Ecuador: Pichinca, alt. 3350 m., March, 1864, *Jameson* (U. S. Nat. Herb.); vicinity of Santa Rosa de Cañar, September 14, 15, 1918, *Rose & Rose 22669* (U. S. Nat. Herb. and Gray Herb.).

The specimens here cited agree in all essential characters with the original description of the above species, although in some respects they correspond equally well to *S. pimpinellaefolius* HBK. In foliar characters, however, the material at hand seems to accord more closely with *S. nubigenus*. Further collections, upon comparison with the types, may prove the two species, as characterized by Kunth, to be conspecific.

Senecio organensis Casar. Nov. Stirp. Bras. Dec. 77; Gard. in Hook. Lond. Jour. Bot. 4:127. 1845; Baker in Mart. Fl. Bras. 6^3 320. 1884.

? *Cacalia dichroa* Bong. Mem. Imp. Acad. Sci. St. Petersb. 1: 40. *t. 7*. 1831.

Brazil: vicinity of Itatiaya, July 26-30, 1915, *Rose & Russell 20565* (U. S. Nat. Herb., fragment and photograph in Mo. Bot. Gard. Herb.).

Senecio otophorus Wedd. Chlor. And. 1:98. 1855-57.

Colombia: Páramo de Buena Vista, Huila group, Central Cordillera, State of Cauca, alt. 3000–3600 m., January, 1906, *Pittier 1129* (U. S. Nat. Herb.); dry, open places. Páramo de Ruiz, Department of Tolima, alt. 3500–3700 m., December 16-17, 1917, *Pennell 3005* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.); Laguna de Buitrago, alt. 3100 m., December, 1919, *Bro. Ariste-Joseph A 500* (U. S. Nat. Herb.).

Ecuador: Páramo de Azuay, *Spruce* (Gray Herb.); vicinity of Cañar, September 16, 1918, *Rose & Rose 22758* (U. S. Nat. Herb.).

Senecio patens (HBK.) DC. Prodr. 6:423. 1837.

Cacalia patens HBK. Nov. Gen. & Sp. 4:164. 1820.

Colombia: in swale, "Rosalito," near Páramo de Ruiz, Department of Tolima, alt. 2800–3100 m., December 15-17, 1917, *Pennell 2971* (N. Y. Bot. Gard. Herb., U. S. Nat. Herb., and Mo. Bot. Gard. Herb.).

Senecio pellucidinervis Schz. Bip. ex Baker in Mart. Fl. Bras. 6^3:319. 1884.

Brazil: vicinity of Itatiaya, July 26-30, 1915, *Rose & Russell 20462, 20571* (U. S. Nat. Herb., fragment and photograph in Mo. Bot. Gard. Herb.).

Senecio Pennellii Greenm., sp. nov. Plate 6.

Frutex scandens; caulibus teretibus striatis juventate arachnoideis mox glabratis, brunneis; foliis alternis petiolatis ovatis vel oblongo-ovatis 10–18 cm. longis 4–8 cm. latis acutis calloso-denticulatis basi leviter cordatis vel rotundatis utrinque glabris vel juventate subtus parcissime arachnoideis mox glabratis, nervis utrinque prominenter reticulatis; petiolis 1.5–3 cm. longis; inflorescentiis terminalibus paniculatis pleuricapitatis; capitulis 12–15 mm. altis heterogamis; involucris campanulatis calyculatis; involucri bracteis 8 anguste oblongis vel oblongo-ovatis 5–6 mm. longis 1.5–3.5 mm. latis ad apicem obtusis vel subrotundatis et pubescentibus cetero glabris; floribus liguliferis 8, tubo gracile 3–3.5 mm. longo, limbo plerumque radiato vel nonnumquam irregulariter dentato flavo, floribus disci 25–30, corollis tubulo-campanulatis, flavibus; pappi setis copiosis albidis; achaeniis glabris.

Scandent shrub; stems terete, striate, arachnoid in the young stages, soon glabrate, brownish; leaves alternate, petiolate, ovate to oblong-ovate, 10–18 cm. long, 4–8 cm. broad, acute, callous-denticulate, shallowly cordate to rotund at the base, glabrous on both sides or in the young stages sparingly arachnoid beneath but soon glabrate, prominently reticulate-veined on both surfaces; petioles 1.5–3 cm. long; inflorescence a terminal many-headed panicle; heads 12–15 mm. high, heterogamous; involucre campanulate, calyculate; bracts of the involucre 8, narrowly oblong or oblong-ovate, 5–6 mm. long, 1.5–3.5 mm. broad, obtuse or rotund and pubescent at the apex, otherwise glabrous; ligulate flowers 8, tube slender, 3–3.5 mm. long, limb usually radiate or sometimes irregularly dentate and occasionally bearing reduced stamens, yellow; flowers of the disk 25–30 with a tubular-campanulate, equally 5-lobed, yellow corolla; setae of the pappus copious, white; achenes glabrous.

Colombia: in wet places, foot of Cordillera Oriental, near Neiva, August 1, 1917, *Rusby & Pennell 572* (N. Y. Bot. Gard. Herb., fragment and photograph in Mo. Bot. Gard. Herb.), TYPE.

Specimens apparently representing this species, but with somewhat smaller heads, and intermediate between it and the following variety, were collected along the Camino de Gachetá, Colombia, January 5, 1920, *Bro. Ariste-Joseph 467 A* (U. S. Nat. Herb.).

Var. **gachetensis** Greenm., var. nov.

Stem arachnoid-tomentulose intermixed with multicellular somewhat villous hairs; leaves about 10 cm. long, one-half as broad, persistently subarachnoid-tomentulose beneath; heads about 20-flowered.

Colombia: Camino de Gachetá, January, 1920, *Bro. Ariste-Joseph A 535* (U. S. Nat. Herb., No. 1059490).

This species is related apparently to *S. Urbani* Hieron. but differs in having larger leaves, longer petioles, shorter involucral bracts, and a distinctly white pappus.

Senecio pericaulis Greenm., sp. nov.

Perennis; caulibus verisimiliter scandentibus lignescentibus teretibus striatis brunneis hirtellis plus minusve glabratis; foliis alternis sessilibus amplexicaulibusque anguste ovatis vel oblongo-ovatis 4–8 cm. longis 1.5–3 cm. latis mucronato-acutis calloso-denticulatis vel leviter sinuato-dentatis utrinque aliquantulum crispo-hirtellis plus minusve glabratis, marginibus saepe revolutis; inflorescentiis terminalibus laxe paniculato-cymosis subglabris vel sparse glanduloso-hirtellis; pedunculis 1.5–4.5 cm. longis; capitulis 12–14 mm. altis radiatis; involucris campanulatis, calyculatis; bracteis involucri 13 lineari-lanceolatis vel lanceolato-oblongis circiter 1 cm. longis 1–3 mm. latis acutis vel obtusis glabris vel sparsissime glanduloso-hirtellis; floribus liguliferis 10–13, tubo 5 mm. longo, ligula oblonga circiter 12 mm. longa 3–4 mm. lata pallide aurantia; floribus disci circiter 30, corollis 8 mm. longis sursum sensim ampliatis; pappi setis albidis; achaeniis glabris.

Perennial; stems probably scandent, ligneous, terete, striate, brownish, crisp-hirtellous, more or less glabrate; leaves alternate, sessile and amplexicaul, narrowly ovate or oblong-ovate, 4–8 cm. long, 1.5–3 cm. broad, mucronate-acute, callous-denticulate or shallowly sinuate-dentate, somewhat crisp-hirtellous on both surfaces, more or less glabrate, especially above, margins commonly revolute; inflorescence a terminal subglabrous or sparing-

ly glandular-hirtellous open paniculate cyme; peduncles 1.5–4.5 cm. long; heads 12–14 mm. high, radiate; involucres campanulate, calyculate with few short setaceous bracteoles; bracts of the involucre 13, linear-lanceolate or lanceolate-oblong, about 1 cm. long, 1–3 mm. broad, acute or obtuse, glabrous or very sparingly glandular-hirtellous; ray-flowers 10–13, tube 5 mm. long, ligule oblong, about 1 cm. long, 3–4 mm. broad, pale yellow; disk-flowers about 30, corollas gradually ampliated from the base; setae of the pappus white; achenes glabrous.

Ecuador: mountains, Province of Cuenca, July, 1864, *Jameson,* without number (U. S. Nat. Herb., No. 700136), TYPE; vicinity of Cumbe, September 25, 1918, *Rose, Pachano & Rose 22983* (U. S. Nat. Herb., No. 1023355).

This species is allied to *S. amplexifolius* HBK., from which it differs in having smaller and more or less crisp-hirtellous leaves, a somewhat glandular-hirtellous inflorescence, and fewer flowers in the head.

Senecio pindilicensis Hieron. in Engl. Bot. Jahrb. **19**:65. 1894.

Ecuador: Quito, without number or date of collection, *Jameson* (Gray Herb.).

The specimen here cited agrees in all essential details with the very careful description of the above species by Professor Hieronymus, and the writer has no hesitation in referring it to that species.

Senecio pulchellus DC. Prodr. **6**:421. 1837; Wedd. Chlor. And. **1**:100. 1855-1857.

Cacalia pulchella HBK. Nov. Gen. & Sp. **4**:160. 1820.

Microchaete pulchella Benth. Pl. Hartw. 210. 1845.

M. trichopus Benth. Pl. Hartw. 210. 1845.

Colombia: vicinity of Bogotá, alt. 2895 m., *Hartweg 1162* (N. Y. Bot. Gard. Herb.); in woods between Huambia and Pitaya, Province of Popaya, *Hartweg 1163* (N. Y. Bot. Gard. Herb.); dry páramo, southeast of Guadalupe, near Bogotá, Department of Cundinamarca, October 12, 1917, *Pennell 2377* (N. Y. Bot. Gard. Herb., Mo. Bot. Gard. Herb., and Gray Herb.).

The leaves on Dr. Pennell's specimen here cited are somewhat larger than described by Kunth for *Cacalia pulchella* on which the above species was founded, but in all other regards there is complete accord with that species.

Senecio rudbeckiaefolius Meyen & Walp. in Nov. Act. Nat. Cur. **19**: Suppl. 1, p. 283. 1843; Walp. Rep. **6**:272. 1846-47.

Bolivia: Rio Tapacari, March 19, 1892, *Kuntze* (U. S. Nat. Herb.); Cochabamba, February 28, 1920, *Holway & Holway 343* (U. S. Nat. Herb.).

Senecio sanctae-martae Greenm., sp. nov.

Caulis lignescens verisimiliter scandens; ramis floriferis teretibus striatis brunneis glabris; foliis oppositis sessilibus late ovatis 5–7 cm. longis 3–4.5 cm. latis crenato-serratis vel supremis subintegerrimis acutis vel obtusis basi cordatis amplexicaulibusque utrinque glabris et plus minusve glaucescentibus; inflorescentiis terminalibus corymboso-cymosis parce pubescentibus multicapitatis; capitulis 1 cm. altis heterogamis; involucris anguste campanulatis breve calyculatis; involucri squamis plerumque 13 lineari-lanceolatis 6–7 mm. longis acutis glabris; ligulis anguste oblongis circiter 1 cm. longis flavis; floribus disci 30–35; pappi setis albis; achaeniis glabris.

Stem ligneous, probably scandent; branches terete, striate, glabrous; leaves opposite, sessile, broadly ovate, 5–7 cm. long, 3–4.5 cm. broad, crenate-serrate or the uppermost entire or nearly so, acute or obtuse at the apex, cordate and amplexicaul at the base, glabrous on both surfaces and more or less glaucous; inflorescence a terminal round-topped many-headed corymbose cyme, sparingly pubescent; heads about 1 cm. high, radiate; involucre narrowly campanulate, calyculate with short bracteoles; bracts of the involucre usually 13, linear-lanceolate, 6–7 mm. long, acute, glabrous; ray-flowers 8–10, rays narrowly oblong, about 1 cm. long, yellow; disk-flowers 30–35; pappus white; achenes glabrous.

Colombia: Santa Marta, Province of Magdalena, 1898-1899, *Herbert H. Smith 1977* (Mo. Bot. Gard. Herb., Field Mus. Nat. Hist. Herb., and N. Y. Bot. Gard. Herb.), TYPE.

Although no notes are recorded concerning the plant here described, yet the specimens at hand indicate a more or less scandent habit. The species, however, is very characteristic on account of the opposite, sessile, glabrous, and somewhat glaucous leaves.

Senecio scortifolius Greenm., sp. nov.

Perennis; caulibus verisimiliter scandentibus lignescentibus brunneis striatis glabris; foliis alternis petiolatis ovato-ellipticis 3–11 cm. longis 1.5–6 cm. latis acutis vel obtusis vel raro subrotundatis integris basi cuneatis utrinque glabris coriaceis et in sicco saepe pallido-viridibus; petiolis .5–2 cm. longis; inflorescentiis in speciminibus axillaribus paniculatis glabris vel paululo

puberulentis, pedunculis usque ad 12 cm. longis glabris; capitulis numerosis 7–8 mm. latis radiatis; involucris campanulatis parce calyculatis; involucri bracteis 8 lineari-lanceolatis vel lanceolato-oblongis 4.5–5 mm. longis acutis vel obtusis ad apicem penicillatis ceterum glabris; floribus liguliferis 3–5, tubulo circiter 3 mm. longo, ligula anguste oblonga 3.5 mm. longa flava; floribus disci 11–15, tubulo in limbum sensim ampliatum transeunte; pappi setis albidis usque ad 6 mm. longis; achaeniis glabris.

Perennial; stems probably scandent, ligneous, brownish, striate, glabrous; leaves alternate, petiolate, ovate-elliptic, 3–11 cm. long, 1.5–6 cm. broad, acute or obtuse, or rarely subrotund, entire, cuneate at the base, glabrous on both surfaces, coriaceous, and in the dried state usually pale or dull green; petioles .5–2 cm. long; inflorescence in specimens at hand in axillary glabrous or sparingly puberulent panicles, peduncles as much as 12 cm. in length, glabrous; heads numerous, 7–8 mm. high, radiate; involucres campanulate, sparingly calyculate; bracts of the involucre 8, linear-lanceolate or lanceolate-oblong, 4.5–5 mm. long, acute or obtuse, penicillate at the apex, otherwise glabrous; ligulate flowers 3–5, tube about 3 mm. long, ligule narrowly oblong, 3.5 mm. long, yellow; flowers of the disk 11–15, tube gradually ampliated into the limb; setae of the pappus white, 5–6 mm. long; achenes glabrous.

Colombia: Santa Marta, alt. 1400 m., coll. of 1898-1901, *Herbert H. Smith 2000* (Mo. Bot. Gard. Herb., TYPE; Gray Herb., Phil. Acad. Nat. Sci. Herb., U. S. Nat. Herb., and Field Mus. Nat. Hist. Herb.).

Venezuela: Páramo de Aricagua, Mérida, alt. 2500 m., March 31, 1922, *Jahn 1032* (U. S. Nat. Herb.).

The species here described has many characters in common with *S. ellipticifolius* Hieron., but that species is described as a shrub with glandular puberulent peduncles and bracteoles, and with somewhat shining leaves. *S. scortifolius* from the several specimens at hand presents every appearance of a scandent plant, and the leaves in the dried state are dull green and are doubtless rather fleshy in the living state. In foliar characters *S. scortifolius* suggests *S. epiphyticus* Kuntze of Bolivia, which has a pronounced fuscous pubescence in the inflorescence and on the involucre. It also simulates *S. Tonduzii* Greenm. of Costa Rica.

Senecio sinapoides Rusby in Mem. Torr. Bot. Club **6**:65. 1896.

Bolivia: vicinity of Cochabamba, coll. of 1891, *Bang 1135*

(Mo. Bot. Gard. Herb., U. S. Nat. Herb., and Phil. Acad. Nat. Sci. Herb.); Cochabamba, March 5, 1920, *Holway & Holway 371* (Gray Herb. and U. S. Nat. Herb.).

Senecio Sprucei Klatt in Leopoldina 24:128. 1887.

Peru: near Tarapota, coll. of 1855-56, *Spruce 3926* (Gray Herb.), CO-TYPE.

Senecio stigophlebius Baker in Mart. Fl. Bras. 6^3:321. 1884.

Brazil: vicinity of Itatiaya, July 26-30, 1915, *Rose & Russell 20504* and *20549* (U. S. Nat. Herb., fragment and photograph in Mo. Bot. Gard. Herb.).

The plant of Rose and Russell accords with Sello's No. 2187, a specimen of which is in the Gray Herbarium, which is the type-number of the above species.

Senecio Stuebelii Hieron. in Engl. Bot. Jahrb. **21**:357. 1895.

Colombia: wet mossy páramo, Páramo de Cruz Verde, near Bogotá, Department of Cundinamarca, alt. 3300–3400 m., September 20, 1917, *Pennell 2055* (N. Y. Bot. Gard. Herb., Gray Herb., U. S. Nat. Herb., and Mo. Bot. Gard. Herb.); moist páramo, southeast of Guadalupe, near Bogotá, alt. 3300–3400 m., September 27, 1917, *Pennell 2255* (N. Y. Bot. Gard. Herb., U. S. Nat. Herb., Gray Herb., Field Mus. Nat. Hist. Herb., and Mo. Bot. Gard. Herb.); Páramo de Choachi, near Bogotá, alt. 3700 m., August 8, 1922, *Killip & Bro. Ariste-Joseph 11975* (U. S. Nat. Herb.).

Senecio subdecurrens Schz. Bip. in Bonplandia 4:55. 1856, name only; Wedd. Chlor. And. **1**:109. 1855-57, description.

S. formosus Rusby in Bull. N. Y. Bot. Gard. 4:393. 1907, not HBK.

Colombia: Páramo de Buena Vista, Huila group, Central Cordillera, State of Cauca, alt. 3000–3600 m., January, 1906, *Pittier 1130* (U. S. Nat. Herb.).

Ecuador: without number, coll. of December, 1876, *Andre* (Gray Herb.); also *Spruce 6001* (Gray Herb.).

Bolivia: without locality or date of collection, *Bang 1958* (Mo. Bot. Gard. Herb. and Phil. Acad. Nat. Sci. Herb.).

Mr. Bang's plant was distributed as "*Senecio formosus* HBK.," under which name it may be found in herbaria.

Senecio subglomerosus Greenm., sp. nov. Plate 7.

Frutex scandens usque ad 3 m. altus; caulibus ramisque teretibus striatis fulvo-tomentulosis; foliis alternis petiolatis lanceolatis vel elliptico-lanceolatis 3–10 cm. longis 1.5–3 cm. latis saepe acuminatis acutis integris juventate parce fusco-puberu-

lentis plus minusve glabratis pallido-viridibus, medio nervio venisque immersis et supra persistenter puberulentis subtus aliquanto prominentibus; inflorescentiis terminalibus paniculatis fusco-tomentulosis; capitulis subdiscoideis sed heterogamis circiter 8 mm. altis in glomerulis dispositis; involucris anguste campanulatis calyculatis; involucri bracteis 8–13 lineari-lanceolatis 5–7 mm. longis acutis penicillatis extrinsecus fusco-puberulentis; floribus femineis 3–5 multo reductis, corollis nonligulatis gracilibus tubuliformibus circiter 4 mm. longis subaequaliter 5-dentatis; floribus disci 10–12 hermaphroditis, corollis circiter 6 mm. longis tubulo-campanulatis aequaliter 5-dentatis; pappi setis albidis; achaeniis glabris vel sparsissime hirtellis.

Scandent shrub, 3 m. or less in height; stem and branches terete, striate, tawny tomentulose; leaves alternate, petiolate, lanceolate or elliptic-lanceolate, 3–10 cm. long, 1.5–3 cm. broad, usually acuminate, acute, entire, sparingly tawny puberulent in the early stages, more or less glabrate, pale green, the median nerve and veins immersed and persistently puberulent above, somewhat prominent beneath; inflorescence a terminal tawny tomentulose panicle; heads subdiscoid but heterogamous, about 8 mm. high, disposed in glomerules; involucres narrowly campanulate, calyculate; bracts of the involucre 8–13, linear-lanceolate, 5–7 mm. long, acute, penicillate, externally tawny puberulent; female flowers 3–5, much reduced, corollas nonligulate, slender, tubuliform, about 4 mm. long, subequally 5-dentate; flowers of the disk 10–12, hermaphrodite, corollas tubular-campanulate, about 6 mm. long, equally 5-dentate; setae of the pappus white; achenes glabrous or very sparingly hirtellous.

Bolivia: Coroico, Yungas, September, 1894, *Bang 2459*, (N. Y. Bot. Gard. Herb., TYPE; U. S. Nat. Herb., Gray Herb., and Mo. Bot. Gard. Herb.).

The relationship of this species seems to be with *S. Jelskii* Hieron. from which, however, it differs in having persistent tomentose branches, tawny puberulent leaves, and rayless pistillate flowers.

Senecio subruncinatus Greenm., comb. nov.

S. formosus var. *subruncinatus* Wedd. Chlor. And. **1**:125. 1855-57.

Herba perennis; caule erecto vel adscendente simplici vel superne ramoso .5–1 m. alto glabro vel sparsissime crispo-puberulento striato plus minusve purpurascenti; foliis inferioribus oblanceolatis 5–10 cm. longis 1–1.5 cm. latis obtusis vel submu-

cronato-acutis sinuato-dentatis basi in petiolo contractis, superioribus sessilibus plus minusve amplexicaulibus lanceolatis 2–12 cm. longis usque ad 1.5 cm. latis grosse dentatis vel subruncinatis glabris vel subglabris, eis ad inflorescentias plerumque multo reductis; inflorescentiis terminalibus subcorymboso-cymosis paucicapitatis vel aliquando unicapitatis; capitulis magnis 15–18 mm. altis radiatis; involucris campanulatis calyculatis; involucri bracteis 21 lanceolatis 10–13 mm. longis 1.5–3 mm. latis acutis penicillatis glabris vel parcissime crispo-puberulentis purpurascentibus; floribus ligulatis circiter 21, tubulo 5 mm. longo, ligula anguste oblonga circiter 12 mm. longa 2–3 mm. lata violaceo-purpurea; floribus disci numerosis; pappi setis albidis; achaeniis parcissime hirtellis.

Perennial herb; stem erect or ascending, simple or branched above, .5–1 m. high, glabrous or sparingly crisp-puberulent, striate, more or less purple; lower leaves oblanceolate, 5–10 cm. long, 1–1.5 cm. broad, obtuse or submucronate-acute, sinuate-dentate, contracted at the base into a narrowly winged petiole; upper leaves sessile, more or less amplexicaul, lanceolate, 2–12 cm. long, 1.5 cm. or less broad, coarsely dentate to subruncinate, glabrous or nearly so, those towards the inflorescence usually much reduced; inflorescence a terminal subcorymbose cyme, few-headed or occasionally one-headed; heads large, 15–18 mm. high, radiate; involucre campanulate, calyculate; bracts of the involucre 21, lanceolate, 10–13 mm. long, 1.5–3 mm. broad, acute, penicillate, glabrous or sparingly crisp-puberulent, purple; ligulate flowers about 21, tube 5 mm. long, ligule narrowly oblong, about 12 mm. long, 2–3 mm. broad, violet-purple; flowers of the disk numerous, more than 100; setae of the pappus white; achenes sparingly hirtellous.

Colombia: wet mossy páramo, Páramo de Cruz Verde, near Bogotá, Department of Cundinamarca, alt. 3300–3500 m., September 20, 1917, *Pennell 2051* (N. Y. Bot. Gard. Herb., U. S. Nat. Herb., and Mo. Bot. Gard. Herb.); Canoas, July, 1917, *Bro. Ariste-Joseph A 87* (U. S. Nat. Herb.).

The writer feels confident that this is the form which Weddell briefly characterized as *Senecio formosus* var. *subruncinatus*; but the slender lower part of the stem, the oblanceolate to subruncinate leaves, and the glabrous or essentially glabrous character of the entire plant seems to warrant its specific rank.

Senecio sylvicolus Greenm., sp. nov. Plate 8.

Frutex scandens; ramis ramulisque brunneis striatis glabris;

foliis alternis petiolatis elliptico-oblongis vel ovatis basi cuneatis apice submucronatis integris utrinque glabris supra fusco-viridibus subtus pallidioribus, nervo medio et nervis lateralibus prominentibus; petiolis 5–10 mm. longis canaliculatis glabris; inflorescentiis terminalibus corymboso-cymosis parce crispo-pubescentibus; capitulis pedunculatis heterogamis circiter 12 mm. altis; involucris campanulatis parce calyculatis floccoso-tomentulosis; involucri bracteis 8 lanceolatis vel lanceolato-oblongis circiter 6 mm. longis 1–2 mm. latis obtusis penicillatisque; floribus liguliferis 5, ligulis anguste oblongis 10–12 mm. longis 5-nervatis flavibus; floribus disci 12–15; pappi setis albidis; achaeniis glabris.

Stem scandent; branches and branchlets brownish, terete, striate, glabrous; leaves alternate, petiolate, elliptic-oblong or ovate, cuneate at the base, submucronate, entire, glabrous on both surfaces, dark green above, paler beneath, midrib and lateral nerves prominent; petioles 5–10 mm. long, canaliculate, glabrous; inflorescence terminating the stem and lateral branches in sparingly crisp-pubescent corymbose cymes; heads pedunculate, about 12 mm. high; involucre campanulate, sparingly calyculate with minute bracteoles, floccose-tomentulose; bracts of the involucre 8, lanceolate or lanceolate-oblong, about 6 mm. long, 1–2 mm. broad, obtuse, penicillate; ray-flowers 5, rays narrowly oblong, 10–12 mm. long, 5-nerved, yellow; disk-flowers 12–15; pappus white; achenes glabrous.

Colombia: edge of forest, "Rosalito," near Páramo de Ruiz, Department of Tolima, alt. 2800–3100 m., December 15-17, 1917, *Pennell 2985* (N. Y. Bot. Gard. Herb., Mo. Bot. Gard. Herb., Gray Herb., U. S. Nat. Herb., and Field Mus. Nat. Hist. Herb.).

This species is related to *S. ellipticus* DC. and *S. ellipticifolius* Hieron. From the former it differs in having smaller leaves and a tomentulose inflorescence; and from the latter it differs in the absence of glandular puberulence, longer petioles, and in the absence of conspicuous calyculiform bracts subtending the involucre.

Senecio teretifolius (HBK.) DC. Prodr. 6:420. 1837.

Cacalia teretifolia HBK. Nov. Gen. & Sp. 4:159, *pl. 357.* 1820.

Ecuador: Palmira, alt. 3200 m., August 23, 1918, *Rose & Rose 22311* (U. S. Nat. Herb. and N. Y. Bot. Gard. Herb.); vicinity of Ambato, Province of Tungurahua, December, 1918, *Pachano 19* (U. S. Nat. Herb. and N. Y. Bot. Gard. Herb.); vicinity of Ambato, August 24-26, 1918, *Rose & Rose 22327* (U. S. Nat. Herb.).

Senecio theaefolius Benth. Pl. Hartw. 210. 1845; Walp. Rep. 6:271. 1846-47.

Ecuador: without further data, *Jameson* (U. S. Nat. Herb. No. 534598).

Bolivia: near Bogotá, *Hartweg 1166* (Gray Herb.).

Senecio tolimensis Schz. Bip. ex Wedd. Chlor. And. **1**:98. 1855-57.

Colombia: sphagnum bog, "Balsillas," on Rio Balsillas, Department of Huila, alt. 2100–2200 m., August 3-5, 1917, *Rusby & Pennell 765* (N. Y. Bot. Gard. Herb. and Mo. Bot. Gard. Herb.); near Bogotá, colls. of 1919 and 1920, *Bro. Ariste-Joseph,* without number (U. S. Nat. Herb.); Páramo de Guasca, January 3, 1920, *Bro. Ariste-Joseph A 478* (U. S. Nat. Herb. and Gray Herb.).

Senecio vaccinioides Schz. Bip. ex Wedd. Chlor. And. **1**:99 *pl. 20, fig. A.* 1855-57.

Cacalia vaccinioides HBK. Nov. Gen. & Sp. **4**:161, *pl. 358.* 1820.

Psacalium vaccinioides DC. Prodr. **6**:335. 1837.

Microchaete vaccinioides Benth. Pl. Hartw. 210. 1845.

Colombia: Páramo de Buena Vista, Huila group, Central Cordillera, State of Cauca, alt. 3000–3600 m., January, 1906, *Pittier 1181* (U. S. Nat. Herb.); dry grassy páramo, Cerro de Focha, near Bogotá, Department of Cundinamarca, alt. 3100–3200 m., December 26, 1917, *Pennell 2198* (N. Y. Bot. Gard. Herb.); moist open places, Páramo de Ruiz, Department of Tolima, alt. 3400–3700 m., December 16, 17, 1917, *Pennell 3073* (N. Y. Bot. Gard. Herb.).

Var. **pruinosus** Wedd. Chlor. And. **1**:99. 1855-57.

Cacalia glabrata HBK. Nov. Gen. & Sp. **4**:161. 1820.

Psacalium glabratum DC. Prodr. **6**:335. 1837.

Microchaete glabrata Benth. Pl. Hartw. 210. 1845.

Colombia: grassy páramo, Páramo de Chaquiro, Cordillera Occidental, Department of Bolivar-Antioquia, alt. 3000–3200 m., February 23, 1918. *Pennell 4261* (N. Y. Bot. Gard. Herb.); Alto del Verjón, *Bro. Ariste-Joseph 1-A* (U. S. Nat. Herb.).

Senecio Weddellianus Hieron. in Engl. Bot. Jahrb. **21**:356. 1895.

S. vernicosus Schz. Bip. var. *microphyllus* Wedd. Chlor. And. **1**:94. 1855-1857.

Colombia: moist open places, Páramo de Ruiz, Department of Tolima, alt. 3500–3800 m., December 16-17, 1917, *Pennell*

3002 (N. Y. Bot. Gard. Herb., U. S. Nat. Herb., and Mo. Bot. Gard. Herb.).

Senecio Xanthopappus Klatt ex Hieron. in Engl. Bot. Jahrb. 21:360. 1895.

Colombia: roadside, Sibate, Department of Cundinamarca, October 13-15, 1917, *Pennell 2514* (N. Y. Bot. Gard. Herb., fragment and photograph in Mo. Bot. Gard. Herb.); Bogotá, coll. of 1917, *Bro. Ariste-Joseph A 9* (U. S. Nat. Herb.); Sabana de Bogotá, May, 1923, *G. H. Pring* (Mo. Bot. Gard. Herb.); San Cristóbal, October, 1918, *Bro. Ariste-Joseph A 267* (U. S. Nat. Herb., Mo. Bot. Gard. Herb., N. Y. Bot. Gard. Herb., and Gray Herb.).

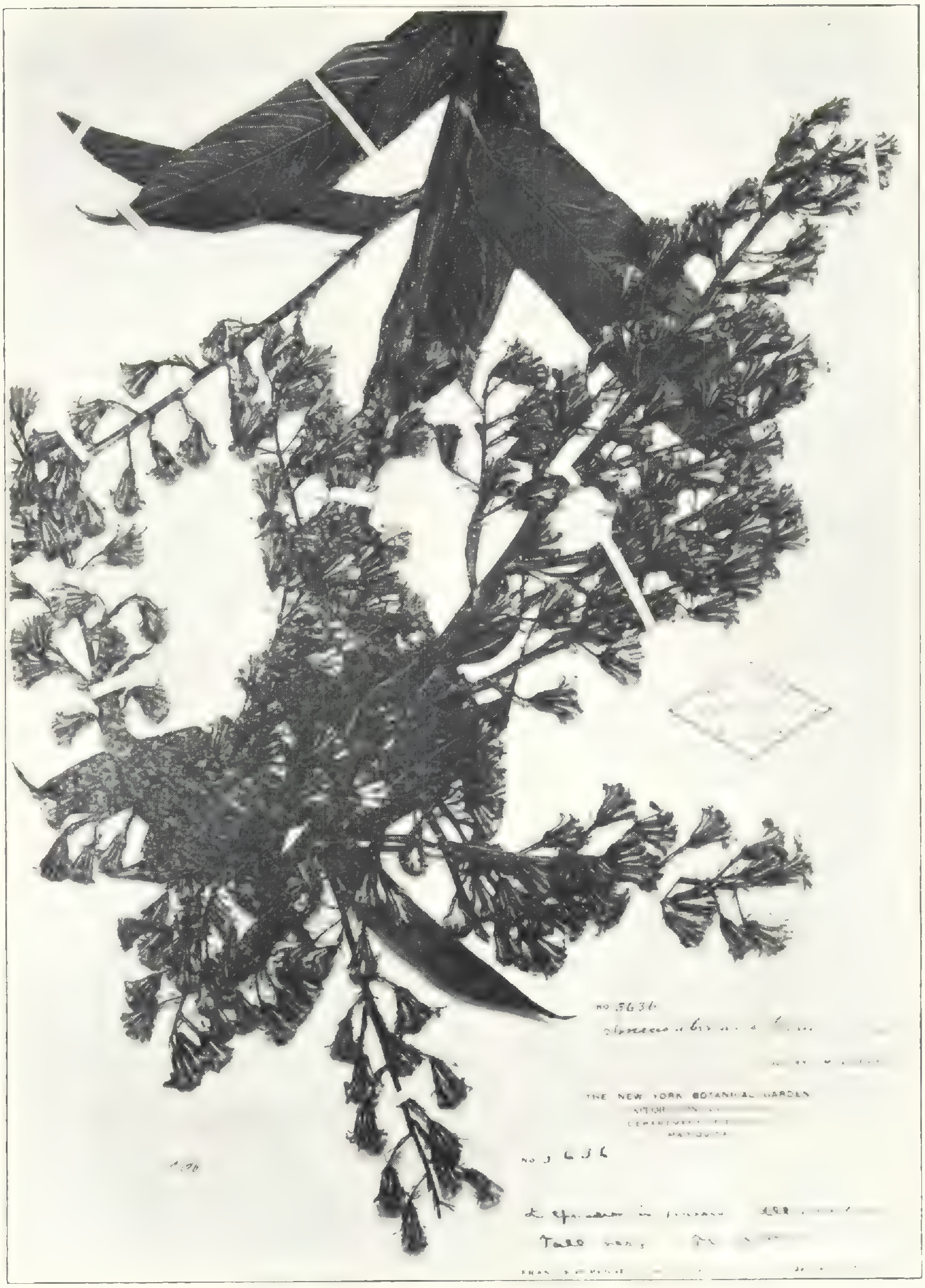
Explanation of Plate

PLATE 3

Senecio aberrans Greenm.

Colombia

From the type specimen, Pennell No. 3636, in the New York Botanical Garden Herbarium.



GREENMAN—SOUTH AMERICAN SENECIOS

Explanation of Plate

PLATE 4

Senecio aridus Greenm.

Colombia

From the type specimen, Pennell No. 3037, in the New York Botanical Garden Herbarium.

GREENMAN—SOUTH AMERICAN SENECIOS

Explanation of Plate

PLATE 5

Senecio lophophilus Greenm.

Colombia

From the type specimen, Herbert H. Smith, No. 1988, in the New York Botanical Garden Herbarium.

GREENMAN—SOUTH AMERICAN SENECIOS

Explanation of Plate

PLATE 6

Senecio Pennellii Greenm.

Colombia

From the type specimen, Rusby & Pennell No. 572, in the New York Botanical Garden Herbarium.

GREENMAN—SOUTH AMERICAN SENECIOS

Explanation of Plate

PLATE 7

Senecio subglomerosus Greenm.

Bolivia

From the type specimen, Bang No. 2459, in the New York Botanical Garden Herbarium.

GREENMAN—SOUTH AMERICAN SENECIOS

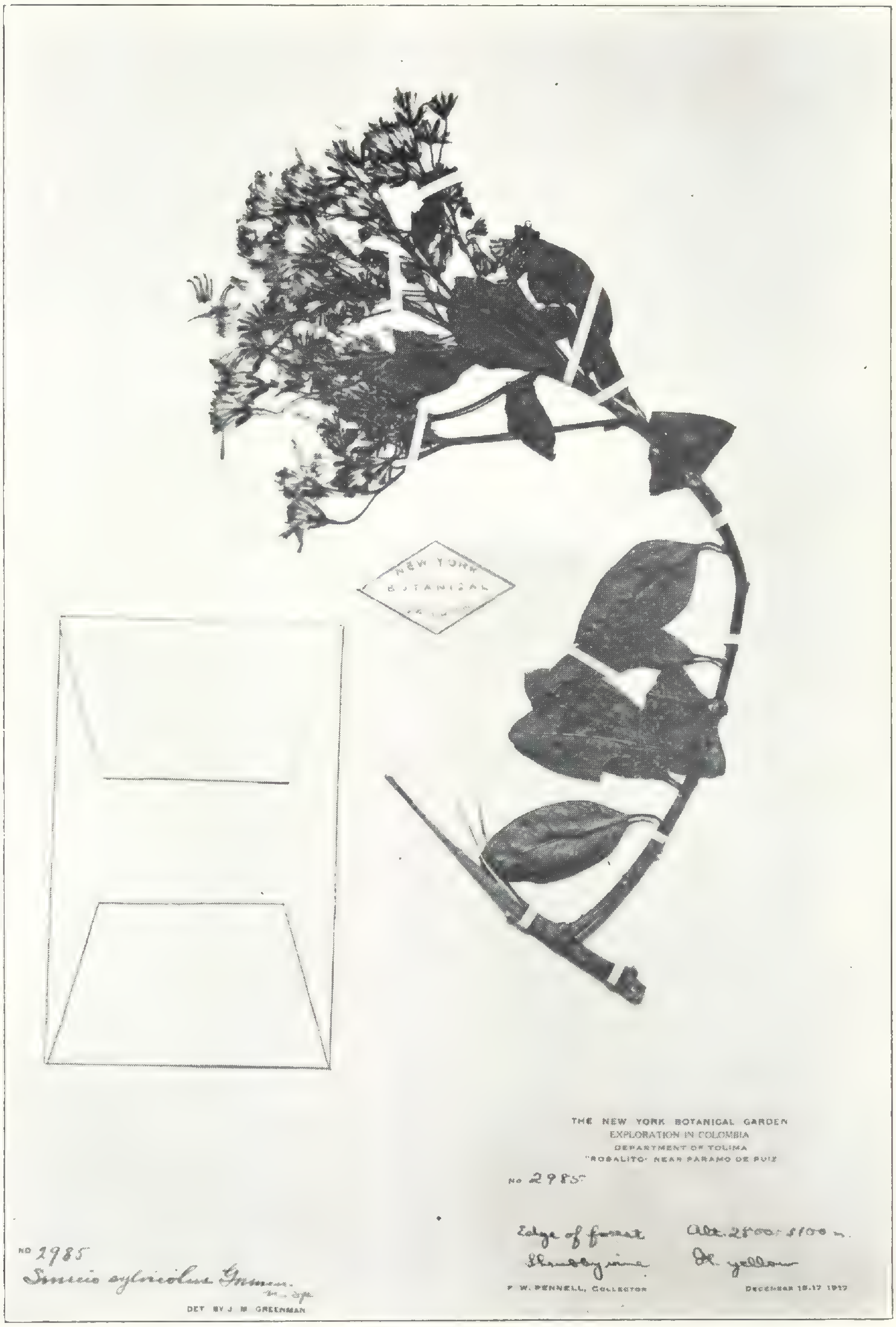
Explanation of Plate

PLATE 8

Senecio sylvicolus Greenm.

Colombia

From the type specimen, Pennell No. 2985, in the New York Botanical Garden Herbarium.



GREENMAN—SOUTH AMERICAN SENECIOS

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POD AND STEM BLIGHT OF SOYBEAN[1]

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This disease was first called to the writer's attention in the summer of 1920. During that season it occurred in abundance on soybeans in the plant-breeding grounds of the North Carolina Agricultural Experiment Station. Later it was briefly described as Phoma blight of soybeans (Wolf and Lehman, '20). The first observations were made about the middle of a somewhat prolonged rainy season of that summer, and at that time comparatively few scattered plants of the early variety Black Eyebrow had been attacked. From the relatively few plants which first succumbed to its attack the disease spread rapidly, first through the remaining plants of the above-named variety, and later to some of the other varieties grown in these plats. Varieties whose ripening processes in the main occurred after the rainy season were lightly attacked.

This disease is known to occur in North Carolina in the counties of Wake, Pender, and Beaufort. It has been reported from no other state, and no reference can be found in available pathological literature to any soybean disease resembling the one described in this paper.

Observations made during 1920 and 1921, seasons very dissimilar in respect to rainfall, indicate that the prevalence of, and

[1] An investigation carried out in part in the department of botany and plant pathology of the North Carolina Experiment Station of the State College and State Department of Agriculture, finished at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy. Published by permission of the Director of the North Carolina Agricultural Experiment Station.

the losses caused by, pod and stem blight of soybeans are closely correlated with rainfall of the summer season. During the rainy season of July and August of 1920 the disease spread rapidly from diseased to adjacent healthy plants, causing considerable damage. With the inception of dry weather a marked falling off of new infections was observed. In the summer of 1921, a season notable for a deficiency in rainfall, very few diseased plants were found, and on these the disease usually involved the stem only near the ground level. Moreover, infection did not spread noticeably to neighboring plants.

The summers of 1920 and 1922 were similar with respect to amount of rainfall. However, during the latter season, the relative humidity was not maintained so constantly at a high point, the rainy period being interrupted by longer periods of drying sunshine. In this season the disease was more abundant than in 1921, but notably less severe than in 1920.

Occasion has permitted no experiments to determine the amount of loss due to this disease. However, it is evident from field observation that, in case of varieties maturing during the wet seasons, very considerable losses occur. This is due largely to the moulding and decay of half-grown seed, and, conservatively estimated, the loss amounted to 15 per cent of the crop from Black Eyebrow in 1920.

Description of Pod and Stem Blight

This disease attacks pods, stems, and leaves. It is seldom found on pods when not also present on some part of the stem of the same plant. Conversely, it is often found on stems when there is no macroscopic evidence of its presence on pods, particularly in dry seasons. The disease makes its appearance first during the warm rainy weather of summer on individual plants standing at intervals among healthy plants. At this time the plants are usually 12–18 inches high and the first pods formed are about half grown. The diseased plants are smaller than the average healthy ones and are usually overlooked by the casual observer. When found, these plants are usually dead, and at least the lower part of the stem bears numerous pycnidia. The infective material spreads rapidly with continuance of rainy

weather, resulting in many more plants becoming diseased as they approach maturity.

On pods.—When infective material is inserted into the wall of the pod, the mycelium spreads in all directions from the point of inoculation, growing throughout the wall tissues of the pod beneath the epidermis. The mycelial advance in the subepidermal tissues is marked by changes which give rise to a watery appearance of infected areas. When infection is more general, such as results from atomizing pods with a suspension of pycnospores, the watery appearance does not develop, but the color of the pod changes to a light brown, which is not greatly different from that of undiseased, ripened pods. In the course of about 10 days of favorable conditions, the entire pod wall becomes invaded, and numerous low, black pycnidia push through the epidermis and soon begin exuding pycnospores (pl. 10, fig. 1). Contemporaneously with pycnidial development, the mycelium invades the seed cavity and attacks the developing seed, often surrounding it with a conspicuous white fungous layer (pl. 12, fig. 1) and penetrating the seed-coat (pl. 9, fig. 3). Pure cultures of the parasite may readily be obtained by carefully breaking open such pods and transferring bits of this mycelial layer from the seed to nutrient agar. Invariably, when numerous pycnidia have developed over the surface of the diseased pods, the seeds, which may have attained nearly mature size, are covered with a more or less conspicuous weft of mycelium, are badly shrunken and wrinkled, and are incapable of germination. Pods are found representing various degrees of this diseased condition. Pods obviously diseased but bearing no surface pycnidia are found with seeds characterized by all degrees of shrinking and wrinkling (pl. 12, fig. 1). Shrunken seeds from such pods almost invariably give pure cultures of the organism causing this disease, and in several instances seeds which were plump and unwrinkled were found to be infected.

Infrequently, the infection may not involve the entire pod, but apparently ceases to advance after invading a small portion of the wall. In such instances, if pycnidia develop at all, they are, of course, confined to the diseased area and are not scattered in characteristic fashion over the entire pod. When very young

pods—up to one-fourth grown—become infected, they usually fall from the plant, while older pods, in which attachment to the stem had become secure before infection occurred, are not shed. Incipient infections sometimes occur which fail to cause general invasion of the pod tissues. Such limited infections are indicated by small brown areas on the pod wall.

On stems.—The disease may be found on any part of the stem and branches. Indeed, the pycnidia often appear on these parts when they are not to be found on the pods. No definite lesions, such as characterize diseases produced by other parasites, are associated with this disease. The mycelium effects a general invasion of the thin-walled portion of the cortex and later enters the stelar tissues, becoming conspicuous in cross-sections of tracheae and pith. After the stem has died or become moribund, pycnidia form in great numbers under favorable conditions (pl. 11, fig. 1B) and are often arranged in rather definite lines extending up and down the stems. This linear arrangement apparently is dependent upon the tendency of the invading organism to grow in the thin-walled chlorenchyma, lying between the more resistant strands of sclerenchyma of the cortex. In wet seasons pycnidia usually appear simultaneously over the entire plant, but in such hot dry seasons as that of 1921 they are usually to be found only on a limited area near the ground. Here requisite moisture for infection and subsequent development is available.

On leaves.—The pod- and stem-blight organism has been found on leaves on only two occasions—once in greenhouse inoculation tests and once in the field. In both instances the type of infection was the same. The fungus is not an active leaf parasite and does not produce the definite spotting such as characterizes the leaf diseases due to *Cercospora*, *Phyllosticta*, and *Phomopsis*, on other leguminous plants. In the case of pod and stem blight of soybeans, leaf infection usually, but not invariably, occurs at the tip or margin of the leaflets and steadily progresses backward from the tip and inward toward the midrib from the margin until the entire leaflet has succumbed, the veins apparently slightly retarding, but not effectually preventing, the advance of the fungus. Invaded laminar tissue loses its characteristic

green color, becomes white, and is soon studded over with small black pycnidia (pl. 10, fig. 2). At the margin of the infected areas the white color of invaded tissue grades into the normal green of uninvaded tissue through a marginal area of 2–4 mm. which is water-soaked in appearance. This discolored marginal area marks the advance of the fungus into non-invaded tissue. Occasionally, infections occur at places remote from the margin of the leaf, and the fungus then advances in all directions laterally, producing the appearance described above.

Morphology

MYCELIUM

The mycelium of the soybean pod- and stem-blight fungus is both inter- and intra-cellular, no aerial growth occurring on host tissue under ordinary field conditions. Plate 9, fig. 2, shows the mycelium growing in the lumina of cells of the stem and penetrating the thick walls of the tracheids. Penetration may occur directly through the wall or by way of cell-wall pits (pl. 9, figs. 4–6). Usually the portion of a hypha actually traversing a cell wall is of much smaller diameter than that of the portion of the same hypha within the cell lumen. Plate 9, fig. 1, shows hyphae within and between cells of the pod wall. In seed cavities of badly diseased pods the mycelium forms a conspicuous white coating over the diseased ovules. Plate 9, fig. 3, shows the fungus growing in the indurated testa of a mature seed.

In culture, the mycelium develops an abundant growth of fine white threads which branch frequently and are rather closely septate. On agar good mycelial growth occurs, and black stromatic masses form in time against the sides of the tubes or are irregularly disposed over the surface of the colonies. On sterile potato plugs the matted character of the mycelium gives way in places to a loose floccose growth. On sterile soybean stems an abundant floccose growth, which often assumes a yellow-green color in small areas, covers the greater portion of the stem. On sterile petioles this aerial growth is not so abundant as on stems, and it appears only near the point of inoculation, little or no aerial mycelium developing at other places. Pycnidia

usually develop most abundantly on the areas devoid of aerial mycelium.

PYCNIDIA

Pycnidia form in great abundance on infected, dead or moribund pods, stems, petioles, and infrequently on leaves. Their position relative to host tissue is apparently dependent on host anatomy. The cortex of the stem of soybeans consists of (1) an outer portion comprising the epidermis and 2–5 layers of thin-walled chlorenchymatic cells, and (2) an inner portion made up of thick-walled sclerenchymatic cells arranged in broad strands which are separated by relatively few thin-walled cells. The pycnidial initials develop in this outer sub-epidermal layer of thin-walled cells, and the developing pycnidium spreads out laterally to form a lenticular fungal structure. Enlargement, however, is usually more rapid in the longitudinal than in the transverse direction of the stem, and as a consequence the pycnidium becomes boat-shaped rather than truly lenticular (pl. 9, fig. 10). It is broadly elliptical in transverse section and possesses a base flattened as a result of the resistance offered by the underlying sclerenchymatic cells of the cortex to the centrifugal pressure of the developing pycnidium (pl. 9, figs. 7–9). Subsequently, a very short beak forms, rupturing the epidermis and affording a means of escape for the pycnospores. Occasionally, a pycnidium may develop within or below the sclerenchymatic cortical tissue. It then develops irregularly, obviously because of inability to destroy or force these cells apart and make room for normal enlargement.

The anatomy of the pod wall permits a more nearly spherical development of pycnidia. Immediately under the epidermis of the pod there is a one-celled layer of sclerenchymatic tissue, and at a distance beneath this is a second thicker layer of sclerenchyma and vascular tissue, the two layers functioning to open the pod upon its maturity. Between these inner and outer layers of mechanical tissue of the pod wall is a several-celled stratum of parenchymatic elements and scattered vascular bundles. Pycnidial development occurs in this broad stratum of thin-walled cells, and as little pressure is needed to rupture the overlying tissues,

enlargement results in a subspherical fruiting body which slightly elevates the overlying epidermis and sclerenchyma layer and later ruptures these tissues by the development of a short beak.

The pycnidia on leaves are less numerous, more scattered, and more nearly isodiametric in cross-section than on pod and stems. The stromatic thickening of the wall is also less conspicuous on leaves and no definite beak is formed.

The majority of the pycnidia are simple, 1-chambered structures. However, individuals with 2 chambers are not infrequently found (pl. 9, fig. 8). The 2-chambered type apparently results from development of two simple individuals in very close contact. The contiguous walls may remain intact or may partly disappear at the time of spore formation, leaving only vestiges of the partition attached to the side walls. Each chamber, however, develops its own ostiole so that a 2-chambered pycnidium has 2 places of escape for spores.

The wall bounding the pycnidial cavity may be divided into 2 portions: an outer layer whose cellular elements are arranged circumferentially with respect to the pycnidium, and an inner, lighter portion of irregular-cellular arrangement from which the slender hyaline conidiophores arise (pl. 9, fig. 9). The cells of this outer layer are thin-walled, light brown in color, and indistinctly discernible at the base of the pycnidium, but, as this outer layer passes around the pycnidium toward the top, these cells increase in number, become larger, thicker-walled, dark brown in color, and more readily perceptible. At the base of mature pycnidia, this outer portion of the wall is very thin, usually only 2 or 3 cells thick, but this thickness is increased to several cell layers on the top and in the vicinity of the beak. The inner layer of the pycnidial wall is composed of irregularly disposed, dilute brown cells, from the innermost row of which the hyaline conidiophores arise.

Pycnidia range in size upon pods and stems from 82 to 225 $\mu \times 82$ to 375 μ, averaging for 131 measurements 169 $\times$ 228 μ. On leaf tissues they are somewhat smaller, ranging from 120 to 180 $\times$ 135 to 240 μ. On stems and petioles the pycnidia are often found to be arranged in rows up and down the stem, and the dimensions of individual pycnidia are as a rule greatest

parallel to the long axis of the stem. Usually the long dimension is 1¼ to 1½ times as great as the short one. On leaves, many of the pycnidia are isodiametric, but a considerable number may be found which are markedly longer in one direction. Pycnidia open by a pore having a diameter of 15–25 μ (pl. 9, fig. 11), through which pycnospores crowd in a steady stream for several minutes when mature pycnidia are immersed in water.

Pycnidial development has been traced macroscopically in cultures on sterile soybean stems. Small stems were placed in test-tubes containing moist absorbent cotton. These were autoclaved and inoculated at the bases where they were in contact with the moist cotton. Under these conditions, pycnidia first became visible as points of whitish growth which appeared somewhat translucent under a hand lens and by microscopic examination were found to consist of pseudo-parenchymatic tissue. At this stage they possessed a diameter of about 0.2 mm., had formed no spores, and appeared to contain a very small quantity of liquid material. The pycnidium enlarged rapidly and the wall became darkened, finally becoming black in color. Simultaneously with this enlargement, hyphae apparently arising from cells constituting the pycnidial wall covered the pycnidium, causing it to appear white. However, upon close inspection, the black pycnidial wall could be seen through this white hyphal mantle, which, because of its evanescent character, disappeared later, clearly revealing the black color of the pycnidial wall. Spores appeared soon after the 0.2 mm. stage and continued for a period of time as yet undetermined for individual pycnidia. A height of 1 mm. and a basal diameter of half of this may be attained in culture (pl. 11, fig. 1 A-C).

The pycnosporophores are simple, slender, hyaline, non-septate, tapering structures; their length varies from 1½ to 3 times that of the pycnospores (fig. 1). They arise from the inner layer of irregular cells of the pycnidium and constitute a bright band lining its wall. Branched forms are very infrequently found.

Pycnospores are single-celled, hyaline, and usually possess 2 large droplets, guttulae, 1 in each end (figs. 2–3). The droplets are not invariably present and disappear in germination.

In shape, pycnospores are straight, fusiform, commonly rounded at one end and noticeably pointed at the other, but may vary by having both ends rounded or one end drawn out to a long, tapering point so that the entire length is nearly twice normal. Slightly curved forms are found. They vary somewhat in size when taken from different sources as indicated by the following measurements:

From stem of host artificially inoculated 4.5–10 × 1.7–2.6 μ, average 6.27 × 2.20 μ.

From the same stem as above, measurements taken after 24 hours in moist chamber: 5.5–9.6 × 1.8–2.5 μ, average 6.52 × 2.20 μ.

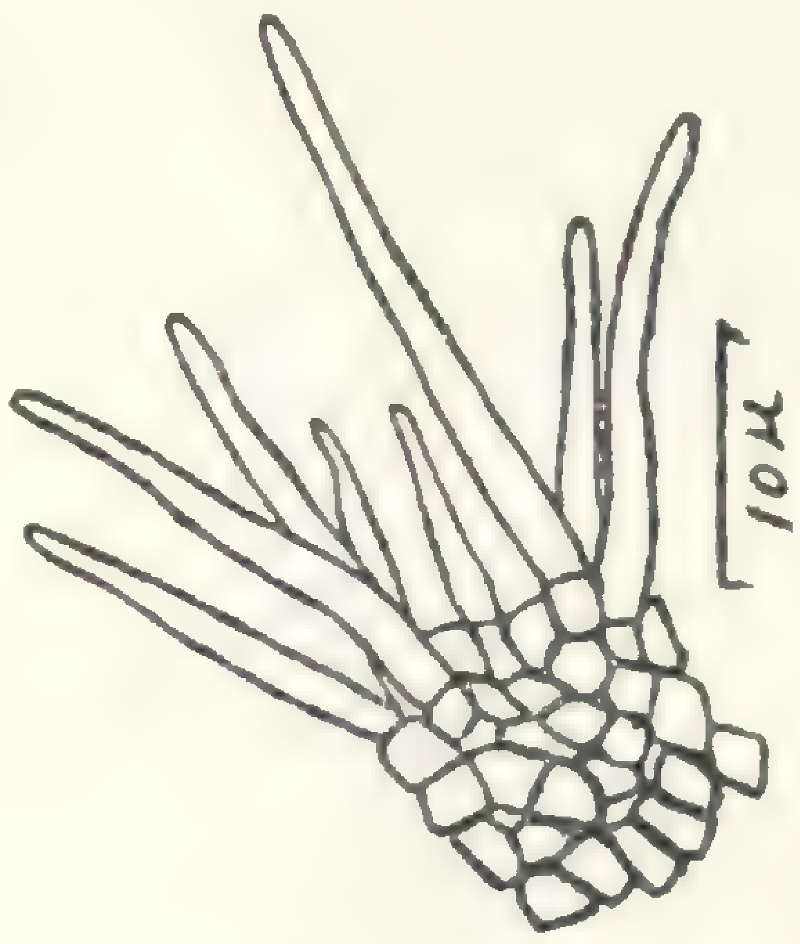

Fig. 1. Pycnosporophores of soybean fungus.

From soybean pod inoculated in moist chamber with spore suspension, measurements made 11 days after inoculation when

Fig. 2. Pycnospores of soybean fungus.

part of pycnidia were exuding spores: 6.0–10 × 1.8–2.8 μ, average 7.15 × 2.29 μ.

From lima bean pod inoculated in moist chambers: 5.5–9.2 × 1.8–2.7 μ, average 6.98 × 2.31 μ.

From soybean leaf 16 days after inoculation: 5.3–8.5 × 1.8–2.8 μ, average 6.57 × 2.30 μ.

From culture on soybean stem 15 days after inoculation: 6.0–9.0 × 1.8–2.7 μ, average 6.91 × 2.18 μ.

From cooked sweet potato four months after inoculation: 5.0–7.6 × 1.9–2.7 μ, average 5.79 × 2.28 μ.

The averages noted above were of 50 measurements in each case, and all measurements were made after the pycnidia had reached maturity as indicated by exudation of pycnospores. The spores were mounted in water and an oil-immersion objective was used in measuring. Spores from pycnidia which developed on pods in moist chambers averaged larger than when developed under drier conditions, as on stems in the open greenhouse.

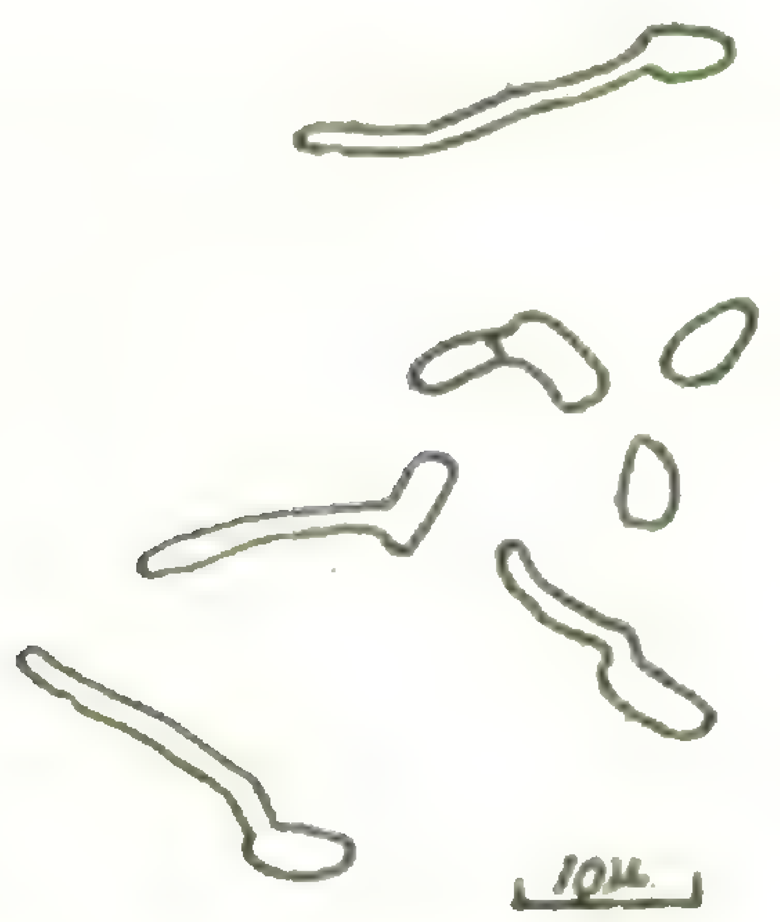

Fig. 3. Pycnospores of soybean fungus, germinating.

As noted above, pycnospores escape through a pore and collect in milky-colored droplets at the tips of the beaks of the pycnidia, from which they are readily splashed by rain. In culture these droplets slowly dry down, becoming yellow and finally some shade of brown. On cultures on soybean stems and petioles, on stems of *Melilotus alba*, and on pods in a moist chamber pycnidia develop and exude spore droplets in 11–13 days when kept at summer temperature in light. If these cultures are prevented from dying out, this may continue for several months. Several cultures on stems of *Melilotus alba*, inoculated August 10, 1921, and kept in a covered glass dish, began exuding pycnidia on the thirteenth day and were still doing so on December 13, 1921, 91 days after inoculation.

Under appropriate conditions, pycnospores begin to germinate in 4 hours after being placed in tap water. The spores take up water and enlarge noticeably, after which one or two slender, hyaline germ tubes are formed. At room temperature during summer, the longest of these tubes may reach a length of 4 to 5 times that of the spore in 18 hours. The guttulae disappear and the tubes continue to grow for about 48 hours. Germ tubes formed on the surface remain long, slender, and sparsely

septate. When germination of spores immersed in the water occurs, the germ tubes soon develop septa and the cells swell and branch in an irregular and curious manner.

Stylospores are found infrequently in pycnidia of the soybean pod- and stem-blight fungus. They are long, slender, hyaline, curved or hooked cells which may or may not be found in the same pycnidium with pycnospores (fig. 4). The greater number of the strains of the fungus isolated from diseased soybeans have not formed stylospores in culture. However, they were found once in a few pycnidia of strain 14 and occur regularly in cultures of strain 17. The latter produces perithecia regularly in culture, but pycnospores are less abundant and stylospores more numerous than in imperfect strains. Stylospores are occasionally found under natural conditions on host tissue. All attempts to germinate them have been unsuccessful.

Fig. 4. Stylospores of soybean fungus.

PERITHECIA

The ascogenous stage of the soybean fungus has never been found in the field. Material wintered out of doors during 2 seasons did not develop perithecia nor has repeated examination of diseased stems and pods picked up in soybean fields at various times during winter and spring revealed any perfect stage. Apparently the soybean fungus seldom, if ever, forms asci and ascospores under such field conditions as exist at Raleigh, North Carolina. Up to the present time perithecia have been found only in culture.

Within the extended period during which this disease has been under observation, the causal organism has been repeatedly isolated from diseased seeds, pods, and stems. Most of these strains have been kept in culture for 6 weeks or more, while a

number have been continued from the beginning of the work to the present time. Strain 17, isolated from a diseased pod in August, 1922, did not differ in general appearance from previously isolated strains, but it was carried in culture because it was early found to produce stylospores more abundantly than any previously isolated form. In September the writer temporarily moved to St. Louis, Missouri, taking with him cultures of several strains. By early November the original, as well as certain subcultures of strain 17, were observed to have formed mature perithecia and ascospores. Examination of 5 other strains, Nos. 7, 11, 14, 19, and 20, showed that perithecia had not yet developed in any of these. In March, however, it was discovered that strain 18, which had been overlooked in the previous examination, had also formed ascocarps in every way similar to those of No. 17. Strains 17, 18, 19, and 20 were isolated at the same time but from different plants, 17 and 18 arising from diseased pods and 19 and 20 from vascular tissue at the lower portion of diseased stems.

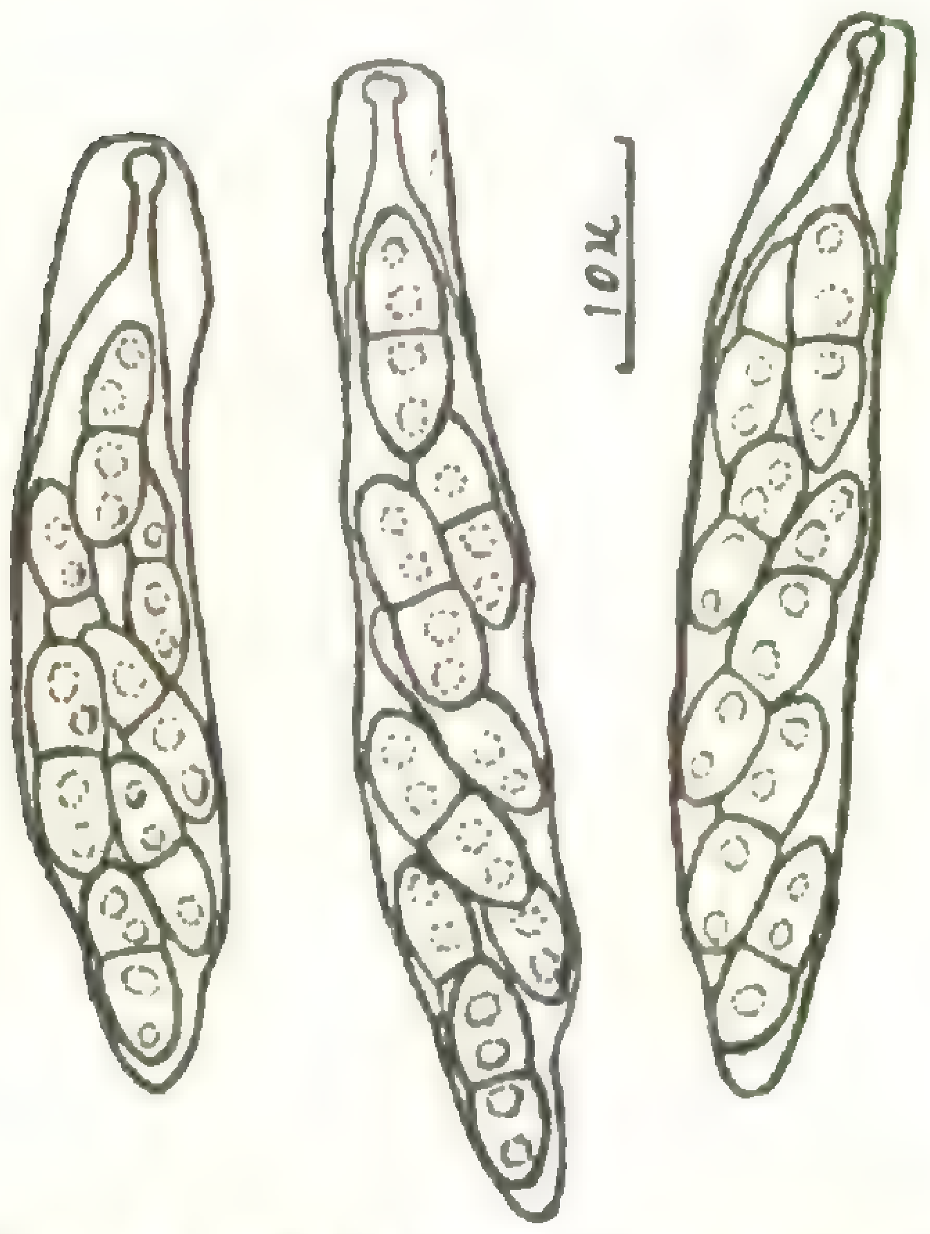

Fig. 5. Asci of soybean fungus, containing ascospores.

Transfers from ascospore strains start off in a manner very similar to those from imperfect strains. Pycnidia bearing pycnospores mature in the course of 14–16 days, but the spores are somewhat less abundant than in cultures of imperfect forms. The pycnidia persist for an indefinite period and are then rather suddenly replaced by perithecia. These fruit bodies may appear in culture in as short a time as 41 days after inoculation when kept at laboratory temperatures during the months of February and March.

In cultures on sterile soybean petioles, the most favorable substratum found by the writer, the perithecia are black in color and are rendered conspicuous by the tendency to form

in clusters of 3–15 individuals, each of which possesses a long, slender, black, crooked or curved beak (pl. 11, fig. 2). The beak often slightly exceeds 1.5 mm. in length, possesses a diameter of 40–60 μ, and is pierced longitudinally by a pore, which presumably serves as an avenue for spore discharge. The bodies of the perithecia are immersed in a stroma. This stroma possesses a dense, black cortical region which covers the perithecia and certain light-colored and less dense stromatic areas (pl. 13, figs. 1–2). The perithecia become spherical when they develop singly, but owing to the fact that several commonly develop within a single stroma individuals are usually flattened in one or more directions by mutual compression. Sizes, which can be determined only from sections of fruiting stromata and which must vary with the amount of crowding, range from 145 to 348 × 116 to 318 μ. Each pycnidium is separated from the stroma by a wall consisting of an outer layer of dark cells and an inner thicker layer of hyaline cells from which the asci arise (pl. 12, fig. 2). The cells of the inner layer are larger than those of the outer and the elements of both layers are arranged circumferentially with respect to the perithecium. The asci are clavate or oblong, sessile, very numerous, vary in size from 37.2 to 50.2 × 7.2 to 12.0 μ (average 44.9 × 8.3 μ) and contain irregularly biseriate spores (fig. 5). The ascus wall is so thin and hyaline as to be difficult to see when unstained, except at the apex where it is markedly thicker and pierced by a narrow pore. Since this pore penetrates a very much thickened portion of the ascus wall and is of very much smaller caliber than the ascospores, it is not apparent that it can well serve as an avenue of escape for them. Presumably ascospores are set free by disintegration or rupture of the thinner portions of the ascus wall. Ascospores do not readily separate from each other in water mounts but adhere in groups, probably by reason of the presence of some adhesive ectoplasmic substance. Ascospores are hyaline, spindle-shaped to elliptical, 2-celled, slightly or not at all constricted at the septum, possess 2–4 guttulae, and measure 9.6–12.4 × 2.4–4.2 μ (average 11.42 × 3.53 μ) (fig. 6). They may begin germination within 4 hours in tap water at 20° C. They swell markedly in thickness and put out germ tubes from one or both cells. The

oil droplets diminish in size and vacuoles of various dimensions appear first in the germinating cell then in the growing germ tube (fig. 7).

Irregularly shaped black stromatic or pseudo-pycnidial bodies are formed in cultures of this fungus. On agar media in plates and tubes they occur at various places over the surface, particularly against the glass sides of the container. When the fungus

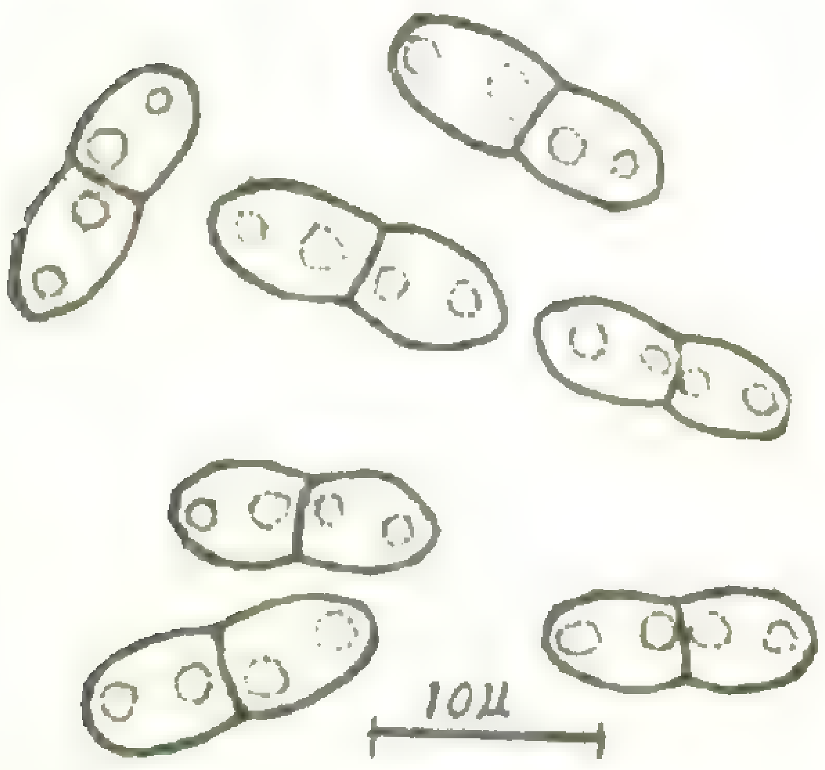

Fig. 6. Ascospores of soybean fungus.

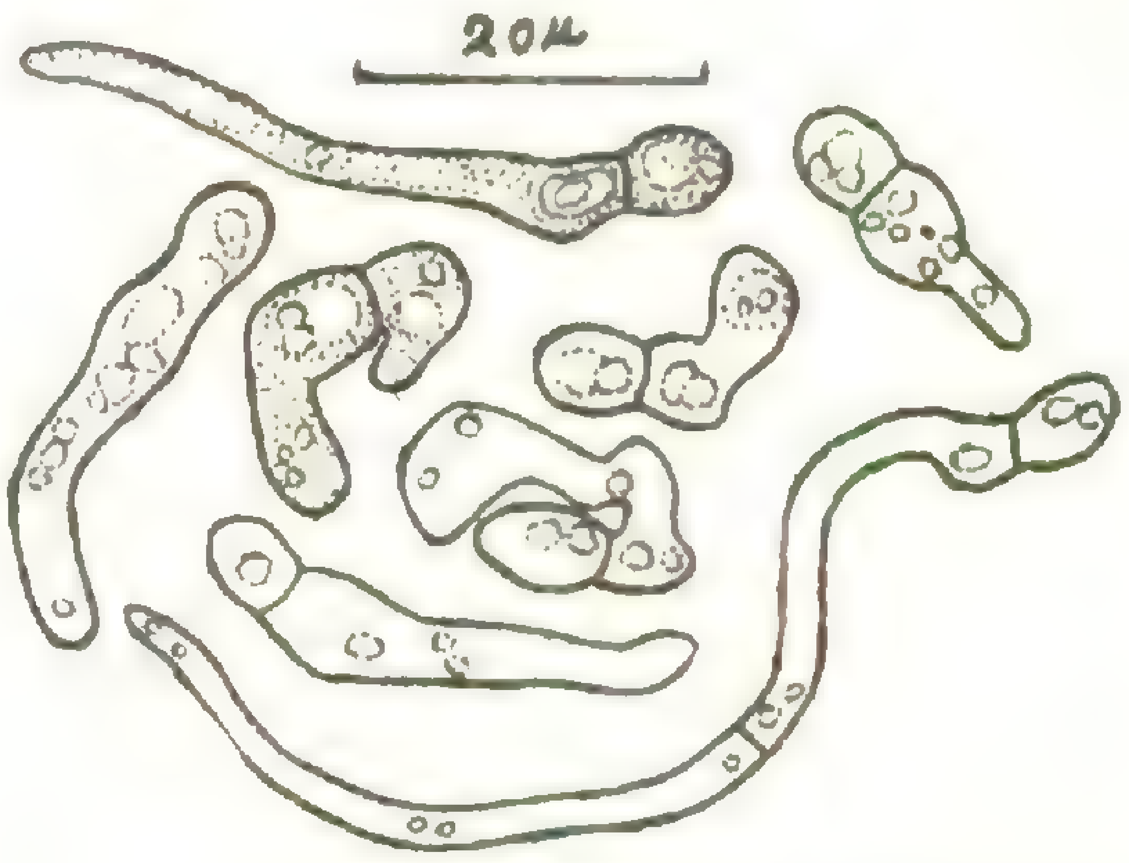

Fig. 7. Ascospores of soybean fungus germinating.

is grown on sterile soybean or sweet clover stems in tubes in the bottoms of which cotton or blotting paper has been placed to hold moisture, the mycelium grows down into this material and forms flat, round, or irregularly shaped stromatic bodies of various sizes. These possess a dense black cortical layer having a thickness up to .4 mm. and enclosing a white or dilute brown aggregation of fungous hyphae closely interwoven with the cellulose fibers of the substratum. In culture, these bodies have developed directly on stems of *Melilotus alba* and were then about the size and shape of a small radish seed. Hand sections show that these bodies possess a cortex of 3 or 4 rows of very dark brown or black cells surrounding a pseudo-parenchymatic tissue of thinner-walled, dilute brown to hyaline elements, the whole resembling a sclerotium in structure. If the cultures are prevented from drying out, one to several pycnidial beaks, which sometimes exude pycnospores feebly, may develop on these stromata. Imperfect strains develop these bodies in a period of time corresponding to that required by perfect strains to form mature perithecia. They resemble in many ways the fertile stromata of perfect

strains and possibly they indicate an abotrive attempt on the part of the former to form perithecia.

The discovery of the perfect stage of a plant pathogenic fungus in culture before the same has been found developing naturally on the host is not without precedent. In 1892, Atkinson ('92) described *Gloeosporium cingulatum* which he had isolated from a diseased stem of privet obtained in New York. No ascogenous stage was present on the host tissue. However, bodies resembling pycnidia or perithecia, but devoid of spores, developed in certain of the cultures. Later Stoneman ('98) obtained perithecia and mature asci in cultures of the same organism isolated from privet sent from Kansas. Still later, Shear and Wood ('13) found fertile perithecia on leaves and stems of privet obtained from Nova Scotia. Shear and Wood ('07) grew both conidial and ascogenous stages of anthracnose fungi from 8 different hosts as follows: *Gloeosporium rufomaculans* from grape, *G. fructigenum* from apple, an unnamed *Gloeosporium* from cranberry, *G. elasticae* from rubber plant, an unreported form from locust, another from *Ginkgo biloba*, *Colletotrichum Gossypii* from cotton, and *C. lindemuthianum* from bean. Of the 8 cases, the perithecial stage of the form from apple only had been found previously under natural conditions upon its host plant. The ascogenous stage of the form from rubber plant was found during the progress of the work reported. Later, Edgerton ('09) found perithecia of the form from cotton on diseased bolls in Louisiana, and named the fungus *Glomerella Gossypii*. In a subsequent paper, Shear and Wood ('13) gave the results of more extensive studies in which they determined the life histories of forms of *Glomerella cingulata* from 36 different host plants. In 17 cases perithecia were produced in pure cultures, and in the remaining 19 cases they developed on the host either in a moist chamber or under natural conditions. In 9 of the 36 cases they were present both in culture and on the host. In 1920, the writer (Wolf and Lehman, '20) found perithecia in cultures isolated from diseased soybean pods which bore numerous *Colletotrichum*-like acervuli. Dr. Shear, to whom cultures were submitted, stated that he believed the perithecia to be those of *Glomerella cingulata*. No perithecia were found

on host tissue. Harter ('13) obtained the ascogenous stage of *Diaporthe batatatis*, a fungus causing a dry rot of sweet potatoes, in cultures isolated from diseased roots. No one has yet reported the finding of perithecia of this fungus on the host.

No reference has been found in available literature to any disease of soybeans agreeing in etiology and pathological symptoms with the one herein described. In 1900 Massalongo ('00) published an account of a leaf spot of *Soja hispida* caused by *Phyllosticta sojaecola* in Italy. The *Soja hispida* to which he referred is probably *Soja max*, but the causal fungi and the characters of the two diseases are different. In 1917, Harter ('17) described a pod blight of lima bean caused by *Diaporthe phaseolorum*, whose pycnidial stage had previously been named *Phoma subcircinata* but which rightfully belongs, as Harter shows, in the form genus *Phomopsis*. Thus it is seen that the lima bean organism in its pycnidial stage, the form commonly found on diseased pods and leaves, falls into the same form genus as the soybean organism. Likewise, the two diseases are similar in general aspect and occur on related genera of host plants. In view of these considerations, it might seem that the two diseases, pod blight of lima bean and pod and stem blight of soybean, are caused by the same pathogen. However, on the basis of the differences noted below, the writer is led to consider the two organisms as distinct species.

1. The stroma associated with the pycnidia of the soybean fungus is notably less well developed than that found associated with the pycnidia of the lima bean organism. In the case of *Diaporthe phaseolorum* the pycnidial stroma is rather extensively broadened and often involves several pycnidia in a single stroma. Likewise, the stroma is well developed beneath the pycnidium, causing its base to appear rather thick. In the case of the pycnidia of the soybean fungus, on the contrary, this stroma is little more than a thickening of the upper portion of the pycnidium and is so sparsely developed beneath as to leave the base of the pycnidium very thin. In fact, the stroma is often entirely absent from the basal part of the pycnidium and the spore cavity is separated from the host tissue by 2–4 rows of indistinctly discernible fungous cells.

2. Pycnidia of the soybean fungus are somewhat smaller than those of *D. phaseolorum.* Harter ('17) finds that pycnidia of *D. phaseolorum* vary in size from 158 to 475 μ, averaging 245.48 μ. Measurements of pycnidia of the soybean fungus on stems and pods vary from 82 to 225 × 82 to 375 μ, averaging 162 × 228 μ. Pycnidia of this fungus are regularly greater in one dimension, usually that which coincides with the direction of the sclerenchyma of the cortex of stem or pod.

3. Pycnospores of the soybean fungus are smaller than those produced by the lima bean parasite. The average size for pycnospores of *D. phaseolorum* is 7.5 × 3.23 μ as given by Harter, while measurements made by the writer from fresh material on pods collected at Raleigh and Willard, North Carolina, average 7.85 × 3.119 μ. Pycnospores of the soybean fungus on stems of soybean average 6.27 × 2.20 μ; on soybean pods inoculated in moist chamber, 7.15 × 2.29 μ; on lima bean pods inoculated in moist chamber, 6.98 × 2.31 μ; on soybean leaves collected from field, 6.57 × 2.30 μ. Thus it is seen that the pycnospores of the soybean organism are not only shorter but also narrower than those of *D. phaseolorum,* the average difference amounting to .7 μ in length and 1 μ in width. When one considers the small size of these spores these differences assume considerable value, amounting to approximately 50 per cent of the diameter of the spores of the soybean fungus. This difference persists when the soybean fungus is grown on lima bean pods.

4. Stylospores are much less frequently found associated with pycnidia of the soybean fungus than with the corresponding stage of *D. phaseolorum.* Only one of the numerous strains of the former isolated from diseased plants has produced stylospores with any degree of regularity, while in the case of the latter, as indicated by the work of Harter, stylospore production may be regarded as the rule rather than the exception on certain media.

5. Pycnidial formation of the soybean fungus is entirely inhibited by keeping the cultures in total darkness. On the contrary, *D. phaseolorum* not only forms pycnidia but produces pycnospores also when kept in darkness.

6. Certain strains of the soybean fungus form perithecia in culture on a variety of media, while no perithecia have ever been found on diseased material from the field. On the contrary, Harter found perithecia on material wintered out of doors but was unable to induce perithecial formation in cultures of the strain derived from ascospores from these perithecia.

For the greater portion of the time during which pod and stem blight of soybean has been under observation only the imperfect stage of the causal organism was known. The pycnidial sporocarps agree very well in point of structure with the description of *Phomopsis* Sacc. as given by Diedicke ('11), except that pycnidia of the soybean fungus are more definitely delimited from the host tissue than is indicated by Diedicke's description and drawings. In citing the differences between the genera *Plenodomus* and *Phomopsis*, he characterizes the pycnidia of the latter as being indistinctly delimited below on account of the hyphal strands pressing between the cells of the host tissue. These are very little in evidence in the case of the pycnidia of the soybean fungus, which in this respect is more like *Plenodomus*. However, the soybean organism is easily separable from the last-named genus by its long slender conidiophores and the distribution of brown color throughout the pycnidial wall. It differs from the form genus *Phoma*, to which it was at first tentatively referred, by reason of the thickened stromatic character of the pycnidial wall. The appearance of perithecia in cultures isolated from diseased pods and the demonstration of the pathogenicity and genetical unity of the two stages render the question of the proper position of the pycnidial stage among the imperfect form genera of only passing importance. On the basis of the morphology of the perithecia the soybean fungus may properly be placed in the ascomycetous genus *Diaporthe* Nitschke, and since it is parasitic on *Soja max* (L.) Piper it is assigned the name *Diaporthe Sojae*. A brief technical description is appended.

Diaporthe Sojae, n. sp.

Pycnidia lenticular, subglobose, often flattened beneath, subepidermal or immersed in the cortex, simple or sometimes chambered, osteolate, black, 82–225 × 82–375 μ; beak very

short or none; wall thin beneath, thick sclerotial above, outer layers black, inner layers dilute brown; stroma diffuse or lacking; sporophores hyaline, simple, slender, tapering, 1½ to 3 times the length of the spores; pycnospores hyaline, continuous, usually 2-guttulate, oblong, often fusiform, seldom curved, 6.27–7.15 × 2.18–2.31 μ; stylospores seldom present, hyaline, slender, curved or hooked. Perithecia spherical or mutually compressed laterally, simple, immersed in black stromata, 145–348 × 116–318 μ; beak very long, slender, tapering, 1.5 mm. × 40–60 μ, black; wall definite, outer layer black, inner layer hyaline; asci sessile, elongate, clavate, thin-walled, 8-spored, 37.2–50.2 × 7.2–12.2 μ (average 44.9 × 8.3 μ), apex thickened and pierced by a narrow pore; ascospores hyaline, elongate-elliptical, 1-septate, 9.6–12.4 × 2.4–4.2 μ (average 11.4 × 3.5 μ), slightly or not at all constricted at the septum, possessing 2–4 guttulae. Perithecia found only in culture.

Parasitic on *Soja max* (L.) Piper in North Carolina.

Isolations

Isolations have been made from diseased stems, petioles, pods, seeds, cotyledons, and seedling hypocotyls. Diseased plants bearing pods and seeds were brought into the laboratory and stored in a screen-covered cage. This was used as material for making isolations at various times during the following winter months. Bundles of diseased plants were also stored out of doors. A partial record of the isolations with the date and source is given below.

1. Made on August 20, 1920, by Dr. F. A. Wolf, from a diseased stem. A loopful of pycnospores suspended in sterile water was spread with a waving motion of the needle over the surface of a hardened agar plate. Individual colonies were sufficiently well separated near the end of the stroke to be easily picked from the plate. Growth typical for this fungus on nutrient agar, such as will be described below, developed in a few days.

3. Made September 20, 1920. (a) Pycnospores from pycnidia on a diseased pod were spread on the surfaces of hardened agar plates. (b) The pods were opened carefully and the seeds found to be covered with a white mycelial web. By use of a

sterile needle, bits of this mycelium were transferred to agar plates. (c) The entire seed was removed with sterile forceps and without sterilization planted in agar. Typical cultures developed in all three cases.

5. Made January 3, 1921. (a) Five seeds were selected from a lot which had been shelled from diseased pods in September and stored in a covered dish in the laboratory. The 5 seeds selected were plump but wrinkled on the back and very little off color. Norton and Chenn's ('10) recommendations for seed disinfection were followed. The seeds were soaked in tap water over night, shaken in alcoholic mercuric chloride solution (2 gr. HgCl in 1000 cc. of 50 per cent alcohol) for 4 to 5 minutes, rinsed in 95 per cent alcohol, washed in several changes of sterile water, then planted on potato glucose agar plates. In 4 days 3 of the seeds had germinated and each of 4 was surrounded by a broad colony of white floccose mycelium characteristic of the *Phomopsis* stage of *D. Sojae.* Bits of mycelium from each of the 4 colonies were transferred to sterile soybean stems and petioles. A profuse growth of white cottony mycelium developed on stems and a rather sparse growth on petioles. Numerous pycnidia developed in these stem cultures from each of the 4 seeds and in the original Petri dish cultures. (b) Five seeds from the same lot as these described under "a" just above, but differing from them in being plump, unwrinkled, and not discolored, were similarly disinfected and planted in agar plates. Broad colonies characteristic of the *Phomopsis* stage of the soybean fungus had developed from 2 of these at the end of 19 days.

6. Made January 14, 1921. These seeds were shelled from a plant which had been kept in a wire cage in the laboratory. The stem and pods of this plant bore many pycnidia and most of the seeds were wrinkled and discolored. Four lots of 5 seeds each were selected and treated as follows: (a) These 5 seeds were only slightly wrinkled and faintly discolored on the naturally yellowish areas. When the seed-coats were removed, no discoloration of the embryos was visible. The naked embryos were disinfected by Norton's method and planted in agar plates. Four of these seeds remained sterile, the fifth yielded a fungus which was not the soybean organism. (b) The seeds of this lot

were more conspicuously wrinkled and discolored than those described under "a" above. Discolored areas were plainly visible on the naked embryos which were disinfected by Norton's method and planted in agar plates. Each of the 5 seeds gave rise to a rapidly spreading white growth, forming a colony which was rather high, loose, and floccose to the very margin, but lower and more dense toward the center. When mycelium from these colonies was transferred to sterile soybean stems, pycnidia developed in abundance. (c) The 5 seeds of this lot differed from those described above in being entirely without seed-coat wrinkling or discoloration. They were surface-sterilized by Norton's method with seed-coats intact and then planted in agar plates. Three of these seeds germinated and gave rise to colonies which produced many fine large pycnidia when transferred to sterile soybean stems. (d) Five seeds appearing in every way like those of lot c were put, without removing seed-coats and without disinfection, into large test-tubes (20 × 2.5 cm.), in the bottom of which was moist blotting-paper. In the case of one of the 3 which germinated, the fungus grew back from the seed-coat or cotyledon upon the hypocotyl and there formed pycnidia characteristic of *D. Sojae*. When killed by the fungus the seedling had reached a height of less than 2 inches compared with a height of 5–6 inches attained under such conditions by healthy seedlings.

7. Made January 22, 1921. Two lots of 5 seeds each were shelled from a diseased plant which had been kept in the laboratory since harvest. These seeds were plump and without surface wrinkling or discoloration. One lot was disinfected and planted in agar plates. Three of these seeds germinated and gave rise to a fungous growth which, when transferred to sterile soybean stems, produced a mycelium and pycnidia characteristic of *D. Sojae*. The second lot of 5 seeds was put, without surface sterilization, into large test-tubes provided with blotting-paper moistened with Shive's 3-salt nutrient solution. Of the 3 seeds which germinated, 2 were soon killed by a fungus whose identity was not determined. The remaining seedling bore a cotyledonary lesion. This lesion was cut out, sterilized in mercuric chloride solution, and planted in an agar plate. This lesion gave rise to

a fungus which produced many pycnidia characteristic of the *Phomopsis* stage of *D. Sojae* on sterile soybean stems This strain has been used in subsequent cultural studies and inoculation work.

9. Made February 18, 1921. Five seeds which were either slightly discolored or both discolored and wrinkled were selected from a lot shelled from a plant which bore segregated, pycnidial areas on the stem, but none on the pods. After disinfection, the seeds were put into large test-tubes containing blotting-paper moistened with Shive's 3-salt nutrient solution. Two seeds which failed to germinate soon became covered with a dense growth of fungus resembling that of *D. Sojae* in every way. This was not further tested. Of the 3 seedlings which germinated 1 was killed when the hypocotyl had attained a length of 1½ inches by growth of a fungus downward from the seed-coat and cotyledons on to the hypocotyl. Pycnidia of *D. Sojae* developed on the hypocotyl and seed-coat. Healthy seedlings reach a height of 5 or 6 inches before dying when grown under these conditions.

10. Made March 12, 1921. Five slightly wrinkled and discolored seeds selected from plants in which the stem and pods bore scattered pycnidia were sterilized and put into large test-tubes containing blotting-paper moistened with the 3-salt nutrient solution. At the end of the seventeenth day the seedling from the only seed which germinated had been killed after attaining a height of 2½ inches by growth of a fungus from the cotyledons to the hypocotyl. Pycnidia of *D. Sojae* formed on the stem below the cotyledons.

12. Made April 23, 1921. This strain was isolated by making a poured plate of pycnospores from a pycnidium on a diseased seedling. The seedling grew from an infected seed which after being sterilized was germinated in a large test-tube containing sterile moist blotting-paper.

14. Made February 15, 1922. Five seeds were taken from a diseased plant which had been collected on September 1, 1920, and stored in a wire cage in the laboratory. These seeds were disinfected by the method recommended by Norton and placed on moist sterile blotting-paper in large test-tubes. One seed did not germinate but gave rise to a fungus which formed stro-

matic bodies on the blotting-paper. When transferred to sterile soybean petioles, pycnidia and stromatic bodies characteristic of *Diaporthe Sojae* were formed. Later stylospores were found in one of these cultures.

15. Made September 15, 1921. This isolation was made from a plant with yellowing leaves and pods about half mature. About 5 inches above the ground was a darkened stem segment which was apparently infected. Hand sections revealed the presence of mycelium in the tissues. The diseased segment was placed in a moist chamber and pycnidia characteristic of *D. Sojae* were formed in great numbers in the course of 5 days. Pycnospores from these were spread on the surface of agar plates and the colonies thus separated were transferred to sterile soybean stems, where a production of mycelium and pycnidia typical of *D. Sojae* ensued.

17. Made August 18, 1922. This isolation was made from a pycnidium from a pod taken from the field on the above date. This is the only strain isolated which has formed stylospores plentifully. It also produces perithecia in culture. When these were first found, single ascus cultures were made for comparative study and use in certain of the inoculation experiments described below.

18. Made August 18, 1922. This isolation was made at the same time and in the same manner as No. 17, but from a pod from a different plant. This strain has also formed perithecia in culture.

From the record of isolations given above, it is seen that the fungus causing pod blight of soybean may be obtained in pure culture from stems, pods, and seeds of diseased plants. That the fungus resides within the seed-coat is beyond question, and it seems highly probable that entrance was effected by actual penetration of the unbroken testa before it had become dry and indurated. Actual parasitism of the embryonic tissues is strongly indicated by the details given for isolations of strains 6 and 7. In the case of strain 6, the only alternative to this interpretation is that hyphae may have grown between the cotyledons in such a manner as to be beyond reach of the disinfectant. However, the presence of discolored areas on the cotyledons is so strongly

suggestive as to almost, if not entirely, cancel the force of the above alternative. Furthermore, the appearance of seeds in pods from the most highly diseased plants leaves little room to doubt that they were killed by the pod-blight organism before reaching maturity.

Attempts to isolate the pod-blight organism from lesions found on cotyledons after seed germination have failed in every case save one. In the spring of 1921, 100 cotyledonary lesions taken from seedlings which had just come through the soil were surface-sterilized and planted in agar plates. All these gave rise to growths of bacteria or *Fusarium* or both; the pod-blight organism was obtained from none of them. The one successful attempt was that of strain 7 described above, in which case the diseased tissue produced the pod-blight organism unaccompanied by bacteria or other fungi. This isolation strongly supports the belief that actual parasitism of the embryonic tissues does occur.

Inoculations

Inoculations have been attempted in the laboratory, the greenhouse, and the field. Field inoculations have not been uniformly successful, due entirely, the writer believes, to the unfavorable influence of the dry weather which prevailed. Preliminary field inoculations were made during the first week of September, 1920. Spore suspension of material from diseased pods was atomized on stems and half-grown pods. The rains which prevailed during the greater part of July and August gave way to dry weather a week or 10 days before these inoculations were made and no infections resulted. The summer of 1921 was remarkable for its deficiency in rainfall, the usual rainy season in July and August failing to develop. A condition approaching serious drought prevailed during the entire growing season for soybeans. Field inoculations were made at 4 different times both by atomizing suspensions of pycnospores on wounded and unwounded plants, and by inserting mycelium into stem and pod tissues. Infection was obtained in one case only. In this instance, the plants were growing on low ground and were large and very bushy, probably maintaining a higher humidity by reason of

their dense foliage. The Haberlandt variety was used for this test. The plants were still green, the pods varying from nearly full size to very small and immature. Sixty-two pods of various sizes and stages of development on one plant were inoculated by inserting mycelium and spores into the wall of the pod, using care not to puncture the cavity of the pod. Thirty-two pods on another plant in the same stage of maturity were similarly wounded but not inoculated. The inoculations were made on August 18 and on September 22, the interval usually hot and dry being marked by subnormal rainfall and high temperatures. Fifteen pods were found with scattered pycnidia over the entire pod or about the wounded area. Others of the inoculated pods which bore no pycnidia on their surface had their seeds attacked and even covered with a mycelial weft. The diseased pods were well distributed over the entire plant. None of the pods on the check plant showed infection, the wounds healing and the pods maturing in an apparently normal manner.

On 4 different occasions, seedlings growing in large test-tubes (20 $\times$ 2.5 cm.) on sterile blotting-paper moistened with Shive's 3-salt nutrient solution have been inoculated by atomizing the seedlings with a water suspension of the pycnospores. Under these conditions, uninfected plants live 4 weeks or more and attain a stem length of 6 inches, pushing vigorously against the cotton plugs and developing the first pair of true leaves. When inoculated with a spore suspension of pycnidia of the soybean pod- and stem-blight organism, the seedlings became diseased and pycnidia developed in large numbers on the stems and cotyledons.

During the summer of 1921, inoculations were made at various times on plants growing in 4-gallon jars in the greenhouse. The results of the most important of these are given in the following paragraphs:

On May 23, 1921, two plants bearing 4 pods each were inoculated by atomizing pods, stems, and leaves with a spore suspension of strain 7. On June 24, 1 pod of each plant was found to be covered with pycnidia. The remaining 6 pods were plucked and put into a moist chamber, whereupon 5 developed many pycnidia in the course of 5 days, the sixth pod remaining free.

The pod-blight organism was reisolated from each of these plants. A third plant bearing half-grown pods was inoculated on the same date by inserting mycelium of strain 7 into the wall of the pod. After 5 days under the bell jar infection was apparent on inoculated pods. Around the point of inoculation there was plainly visible a circular area of darkened tissue, 8 mm. in diameter, bearing whitish tufts of mycelium on the surface.

On May 28, 2 plants 18 inches high and bearing several pods each were inoculated by inserting pycnidia of strain 7 into the pod walls of one plant and by laying the inoculum on the surface of the pods of the other. The plants were then covered with a bell jar. On June 7, infection was apparent on 2 of the wounded pods. The causal organism was isolated and found to be identical with that in the original cultures. On July 12, the 4 remaining pods of this plant were brown and apparently mature. However, upon being put into moist chambers, all 4 developed pycnidia. On June 29, 2 of the pods inoculated by placing pycnidia on the unwounded surfaces gave evidence of infection as shown by failure to fill out and by discoloration. The causal organism was reisolated from the interior of these pods. A few days later other inoculated pods on the same plant developed surface pycnidia. In all, 4 of the 6 inoculated pods of this plant became infected.

On July 7, Pots 13 and 16, each containing 3 plants, were inoculated with strain 12. In each jar one plant was inoculated by inserting mycelium into the stem and the wall of the pods, a second plant by laying inoculum on unwounded pods, and the third by rubbing a spore suspension on wounded pods. Pot 13 was covered with a bell jar for 4 days and Pot 16 was left uncovered. By July 22, 3 pods on plants of Pot 13 inoculated by inserting mycelium, and 2 pods on a plant inoculated with a spore suspension, had become infected. In Pot 16, infection was evident on 5 pods of the plant inoculated by inserting mycelium into the wall of the pod. Cultures reisolated from one of these pods did not differ from those of the strain used for inoculation.

On June 25, inoculations were made as follows: Pot 21 contained 1 plant with 18 pods varying in length from 1 to 2¼ inches, the oldest being apparently full-size but still green.

Pods, leaves, and stems were atomized with a spore suspension. Pot 19 contained 2 plants bearing 15 pods in the same state of maturity as the plant in Pot 21. Pods, leaves, and stems were atomized with a spore suspension. Pot 10 contained 1 plant bearing 11 pods, the oldest being still green but just at the point of turning brown. All were atomized with a spore suspension. Pot 17 contained 2 plants; one with 8 pods was inoculated by inserting mycelium into pod walls; the other, by laying mycelium on unwounded pod. Pot 18 containing 2 plants with 20 pods was used as a check, a part of the pods being wounded by pushing tweezer point through wall. Strain 13 was used for these inoculations. The plants were covered with bell jars and shaded from direct sunlight. By July 1, infection was apparent on all wounded pods of Pot 17, pycnidia being present on most of them. One unwounded pod showed definite infection. The bell jar was removed on this date. By July 7, 3 unwounded pods of Pot 17 had become infected. No infection was apparent on stems or pods of Pots 21, 18, and 10, but the leaves of these plants possessed marginal areas of dead tissue dotted above with many pycnidia. It was very apparent that the causal organism was advancing into uninvaded tissue. The bell jar was removed from Pot 10 on this date. By July 13, pycnidia were present on a few and infection was evident on most of the pods of Pots 19 and 21. The stems and most of the petioles were still green but most of the leaves had fallen. Pycnidia were numerous on stems in Pot 10, but none were present on pods. The plants of Pot 18 were entirely free from disease at this date.

On June 27, the plants in 2 more jars were inoculated. Pot 31 contained 2 plants. The older of these was beginning to mature, as indicated by the formation of yellow-brown color in the 12 pods and in the leaves. The younger plant had 23 pods, the largest being full-size but still green. The leaves likewise were green with no indication of turning. These plants were inoculated by spraying with a spore suspension. Pot 27 containing 2 plants, one with 9 yellowing pods, the other with 9 green pods, with no indication of yellowing, was inoculated by placing mycelium on unwounded pod walls. The plants of both pots were kept under bell jar and shaded from direct sunlight.

A reisolation of strain 7 was used as a source of inoculum. By July 7, pycnidia were numerous on stem, petioles, and 2 pods of the older plant of Pot 31, while the younger plant showed no infection of any part. The bell jar was removed from this pot at this date. In Pot 27, the oldest pod bore pycnidia and infection was evident on 2 other pods, while no infection was manifest on the younger plant. By July 13, 8 pods of the older plant in Pot 31 bore pycnidia. No pycnidia were present on pods of the younger plant, but numerous small browned spots, points of infection, were visible. In Pot 27, the older plant had shed all its leaves and pycnidia were present on the stem, petioles, and all the pods. The younger plant maintained the green color of its leaves and infection was apparent on 3 pods but no pycnidia had yet developed.

On September 22, 2 plants bearing 7 pods each were inoculated by inserting pycnidial material of strain 7 into the pod walls. The plants were then covered with bell jars for 2 days. By the end of the seventh day, 8 of these pods gave visible evidence of infection. The plants were destroyed by rats before further observations could be made.

Inoculations have been made at various times by placing pods ranging from very young to full-grown and mature in moist chambers and spraying them with suspensions of spores. Under these conditions pycnidia develop on all pods regardless of stage of maturity in from 9 to 12 days.

In order to test the pathogenicity of the ascospore-producing strain, it was necessary to use cultures arising directly from ascospores. By following the usual procedure, cultures of strain 17 were obtained which are known to have arisen from a single ascus. On March 11, 1923, inoculations were made on the pods of plants growing in the greenhouse at the Missouri Botanical Garden. These pods varied in the state of maturity from those in which the ovules had not begun to swell to those which were fully half mature and 1½ inches long. Inoculations were made by inserting crushed perithecia into the tissues of the pod wall, using due precaution not to puncture through into the cavity. The plants were covered with newspaper for 48 hours to prevent the wounds from drying before the fungus could start growth.

Seven days after the time of inoculation, 6 of 11 pods inoculated showed definite infection, the fungus having grown through the pod and killed the tissues opposite the point of inoculation. By the end of 14 days from the time of inoculation, 9 pods showed infection and one had formed pycnidia on the diseased area. The fungus was reisolated from these diseased pods and behaved in every way like parallel cultures started from the tube cultures used as the source of inoculum. Inoculations made on the same date as those mentioned above, but with the imperfect strain No. 14, yielded at the end of 14 days 9 infected pods out of 9 inoculated. No difference was apparent in the appearance of diseased pods and the course of the disease resulting from inoculation with the two strains.

In a second test, 24 pods were inoculated with the ascospore strain, No. 17, and 17 pods with the imperfect strain, No. 14. By the end of 8 days, 10 of the former and 11 of the latter showed definite signs of infection. At the end of 15 days, 19 of the former and 16 of the latter were diseased.

By way of summary of the foregoing record of inoculations, it may be said that infection can be accomplished even under adverse conditions of temperature and moisture by inserting the inoculum into the wall of the pod. This method is almost invariably successful where average atmospheric humidity obtains in conjunction with average summer temperatures. Atomizing with spore suspensions does not result in uniformly successful infections except when, by natural or artificial means, a relatively high atmospheric humidity is maintained for several days. Such conditions obtain during our usual summer rainy season and may be approximated, although unsatisfactorily so from the standpoint of host reaction, by the use of bell jars in the greenhouse. The results of these inoculations, considered in conjunction with the isolations detailed above, furnish conclusive evidence that the organism herein described and named *Diaporthe Sojae* is the cause of soybean pod blight.

Manner of Infection

The exact manner by which hyphae of the fungus causing pod and stem blight of soybean enter the plant has not been fully

determined. As in the case of other plant parasites, entrance may be effected through wounds produced by insects, such as leaf-hoppers and flea-beetles, which puncture the epidermis in their feeding operations. It would seem, however, judging from the general infection of many plants, that the fungus is not dependent entirely on wounds for access to the host tissues. Stomata present on pods and leaves doubtless serve as places of entrance. Moreover, the hyphae may be able to penetrate the unbroken epidermis. It has not been possible to date to determine this point with certainty.

The difficulty of obtaining infections under dry conditions when spores are atomized on pods and stem would indicate that entrance is mainly through the stomata. Infections are readily obtained by this method when plants are maintained in a highly humid atmosphere, possibly because stomata are usually open when light and atmospheric moisture are abundant.

Infection is first made evident on pods by the appearance of a darkened, water-soaked area about the point of inoculation or by a premature yellowing and browning of infected tissue. Pycnidia may or may not appear on the surface. Even when pycnidia do not develop on diseased pods in the open, the ovules or seeds may be found covered with a conspicuous weft of hyphae, and the characteristic fruiting bodies of the fungus develop when such pods are placed in moist chambers. If pods become infected when less than about one-fourth grown, the ovules commonly fail to develop further and the pods fall from the plant. Older pods usually cling firmly to the stems bearing them.

Overwintering and Dissemination

The soybean pod-blight organism passes the winter on dead stems and pods and in diseased seed. Dead stems bearing numerous pycnidia were collected in the fall and wintered out of doors on the ground. Pycnospores were present in a part of the pycnidia on May 5 of the ensuing year and these showed abundant germination in tap water at room temperature. Stems similar to those described above were wintered in an open can in the laboratory. On May 18 spores from these stems

produced vigorous germ tubes when placed in tap water. It is not known how long an individual pycnidium may continue to produce spores under favorable conditions. In cultures on stems spore production is usually terminated by loss of moisture from the substratum. Cultures on stems of *Melilotus abla,* made August 10 and kept in a covered glass dish to retard drying, were still sporulating on December 22. It is very probable that the same pycnidium may sporulate abundantly in the fall, remain dormant during the unfavorable temperatures of winter, sporulate again when favorable conditions return, and thus constitute a source from which the new crop may become infected.

The fact that the causal organism may be isolated from seeds suggests that this is a means of overwintering. Badly infested seeds fail to germinate; others less severely injured germinate but soon after are killed by the parasite. Pycnidia formed on the dead seedlings sporulate during moist weather and thus constitute a source of inoculum from which healthy plants may become infected.

Dissemination of this disease comes about for the most part through infected seed. Much of the infected seed is badly shrunken, light, and non-viable. Most of this will be removed in the cleaning process. Other seeds which become infected at a later stage of development differ very little in weight and appearance from healthy seed and are still viable. This class of seed is the chief means of dissemination of this disease over long distances. Wind, rain, and insects all probably serve to spread the disease from plant to plant.

Varietal Susceptibility

Of the 3 seasons during which pod and stem blight of soybean have been under observation, in those of 1920 and 1922 only did the disease appear in the field with sufficient prevalence to constitute a basis of observation of varietal susceptibility. In these years, the variety known as Black Eyebrow was attacked first and suffered more severely than others. Austin and Haberlandt were next in point of damage done, although these two varieties were much more lightly attacked than Black Eyebrow. It seems probable, however, that the greater damage done to

Black Eyebrow may not depend so much on greater lack of inherent resistance of this variety compared with other varieties as upon the coincidence of favorable weather conditions for development of the disease and favorable state of maturity of the plants. Infections are most readily accomplished when plants are nearing maturity, and this condition obtained for the Black Eyebrow soybean as the rainy season approached an end. Other varieties, Mammoth, Medium Yellow, Virginia, Wilson, Tar Heel Black, Arlington, Chiquita, Brown, and Tokyo have suffered no damage from this disease.

Cultural Characters

The fungus causing pod and stem blight of soybean has been grown on a variety of culture media. An abundance of mycelium with few or no fruiting bodies is formed on agar, while on soybean stems mycelium is usually present in abundance and pycnidia are numerous. Soybean leaf petioles commonly produce less mycelium and larger pycnidia than stems of the same plant. Stems of *Melilotus alba* prepared and sterilized when the plants have nearly reached maturity give rise to a very sparse mycelial growth and many large pycnidia. In short, the mycelium is more profuse on agar media and the pycnidia are larger and more numerous on petioles of soybean and stems of *Melilotus alba*. Below is given a brief descriptive account of this fungus as it appears when grown on various substrata. Except in cases otherwise designated, the descriptions apply to test-tube cultures of strains 6, 7, and 14 kept in indirect light at the laboratory temperatures prevailing for the time covered by the dates given.

Stems of Melilotus alba.—Inoculated August 10, 1921, and kept in a covered glass dish. On August 23, mycelium white, very sparse, the brown color of the stem plainly visible through the thin network; pycnidia very numerous, black, no surface covering of short white hyphae as when grown on soybean stems, exuding spores in milky droplets. December 22, pycnidia numerous, large, still sporulating, some standing singly, others aggregated in twos or threes; stromatic masses formed on cotton at the bottom of the tube.

Soybean stem and petiole.—Inoculated March 31, 1921. On April 5, mycelium profuse, loose, high, growing down the stem, white on the petiole and white with yellow-green in small areas on the stem; no pycnidia. April 15, on stem, mycelium loose, high, profuse over entire stem, white except at top which is yellowish; pycnidia just appearing. On petiole, mycelium profuse over upper one-fourth, white with yellow-green areas, remainder of petiole natural brown, mottled with black areas; pycnidia numerous over entire stem, exuding spores in watery white droplets. May 15, same as for previous date except spore droplets dried to brown color.

Inoculated August 18, 1921, with strain P 11 and kept in a covered glass dish. August 23, mycelium profuse on upper half of stems, rather sparse on petioles, white with yellow-green in small areas where profuse; pycnidia numerous on petioles, fewer on stems; no spores exuded. September 22, pycnidia numerous on both stem and petioles, exuding pycnospores which are drying to a yellow or brown color.

Potato plugs.—Autoclaved in special tubes constricted to hold the plug out of water. Inoculated March 31, 1921. April 5, mycelium loose, high, profuse, white, spreading over entire plug. April 15, mycelium profuse, dense, high, white with considerable brown about the point of inoculation. May 15, mycelium densely matted in places, high in others, matted over surface of liquid; black stromatic masses present but no pycnidia. July 20, no pycnidia.

Sweet potato.—Fifty grams of potato sliced and autoclaved in enough water to cover, in 750-cc. flasks. Inoculated March 31, 1921. On April 5, mycelium profuse, moderately dense, white, spreading rapidly. April 15, mycelium covering surface of substratum, greenish, yellowish, with dirty brown areas, droplets of brown fluid on surface. May 16, mycelium dirty brown with white areas; black, brown, and white areas on bottom and sides of flask. Black stromatic masses present but contain no pycnospores. July 21, mycelium a dirty brown with white areas and black stromatic masses. Reverse side of culture mostly black with white areas; very few pycnidia with pycnospores. Pycnospores have characteristic droplets, are some-

what more pointed at one end than at other, but average shorter than on host tissue.

Rice.—Autoclaved 10 gms. rice in 20 cc. tap water in 100-cc. flask. Inoculated March 31, 1921. On April 5, mycelium profuse, loose, high, white. April 15, mycelium profuse, moderately dense, white, covering and permeating substratum. May 15, surface mottled with yellowish, white, and black areas. July 20, surface brown with darker brown areas; reverse with black areas. No pycnidia.

Oats.—Autoclaved 5 gms. rolled oats in 15 cc. tap water in 100-cc. flask. Inoculated March 31, 1921. On April 5 mycelium profuse, loose, high, white. April 15, mycelium moderately dense over surface but not visible in reverse, white, yellow, black, and brown areas, black marginal line against glass sides of flask. May 15, surface with white, black, and brown areas; substratum yellow, black stromatic masses present; no pycnidia. July 20, surface mostly white, but with citron and light brown areas. No pycnidia.

Corn meal mush.—Autoclaved 5 gms. corn meal and 15 cc. tap water in 100-cc. flasks. Inoculated March 31, 1921. April 5, mycelium profuse, dense, moderately high, white. April 15, mycelium white with yellowish and dark areas, black marginal line against glass. May 15, surface of mat with white, black, and yellowish areas; black stromatic masses but no pycnidia present. July 20, no pycnidia.

Potato glucose agar.—Test-tube cultures inoculated March 31, 1921. April 5, mycelium white, loose, growing high up sides of tube. April 15, mycelium matted, white; reverse and marginal line black. May 15, surface white with black areas; reverse very black; no pycnidia. July 20, no pycnidia.

Bean agar (*Harshberger*, *'17*).—Test-tube cultures inoculated March 31, 1921. April 15, mycelium loose, high, white. May 15, mycelium prostrate and appearing sparse; stromatal masses large and numerous; no pycnidia; reverse white. July 20, no pycnidia.

After discovery of the ascospore strain No. 17, it was compared culturally in another series with strains 7, 14, and 19. Aside from minor variations in respect to color changes, No. 17 differs

from the other 3 strains in the presence of asci, numerous stylospores, and less abundant production of pycnospores. By the end of 17 days, all the strains had formed pycnospores on stems of *Melilotus alba,* corn meal mush, and corn meal agar, and had failed to sporulate on potato plugs and cooked rice, strain 17 forming stylospores in addition wherever pycnospores were formed. By the end of 48 days, strain 17 had produced mature

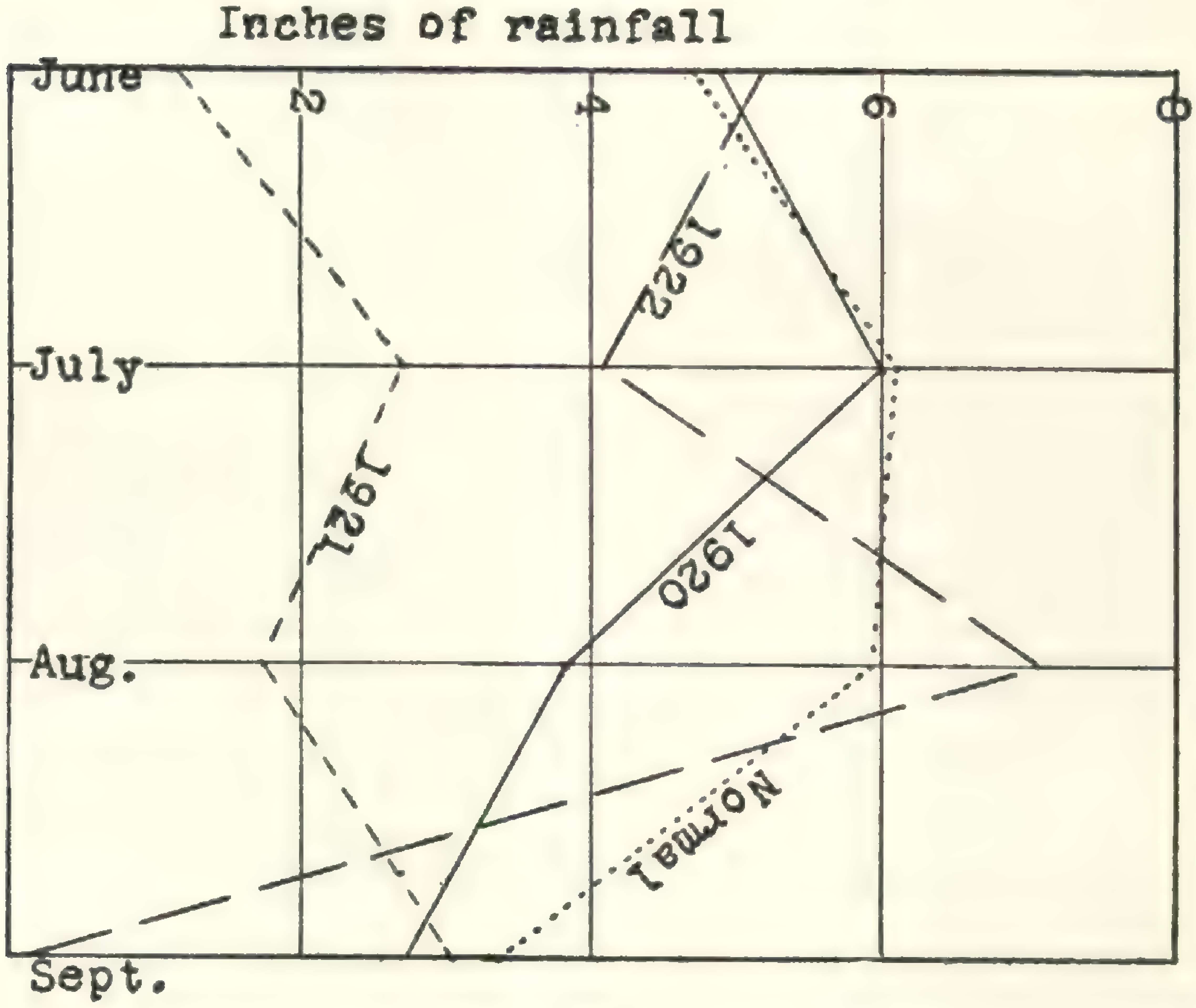

Fig. 8.

perithecia on *Melilotus alba* stems, corn meal mush, corn meal agar, potato plugs, and cooked rice, while the other strains had entirely failed to sporulate on the 2 last-named substrata. As short a time as 41 days is sufficient for the production of mature perithecia in cultures of strain 17 on soybean petiole.

Relation of the Amount of Rainfall to Prevalence of Pod and Stem Blight

Observations made during the summers of 1920, 1921, and 1922 indicate that the prevalence of, and the losses caused by,

pod and stem blight of soybean are correlated with the rainfall of the growing and ripening season. The curves of fig. 8, founded on data collected at the Raleigh station of the United States Weather Bureau, represent normal and total precipitation at Raleigh during the months of June, July, August, and September, of the 3 above-mentioned years. The curve for 1920 shows

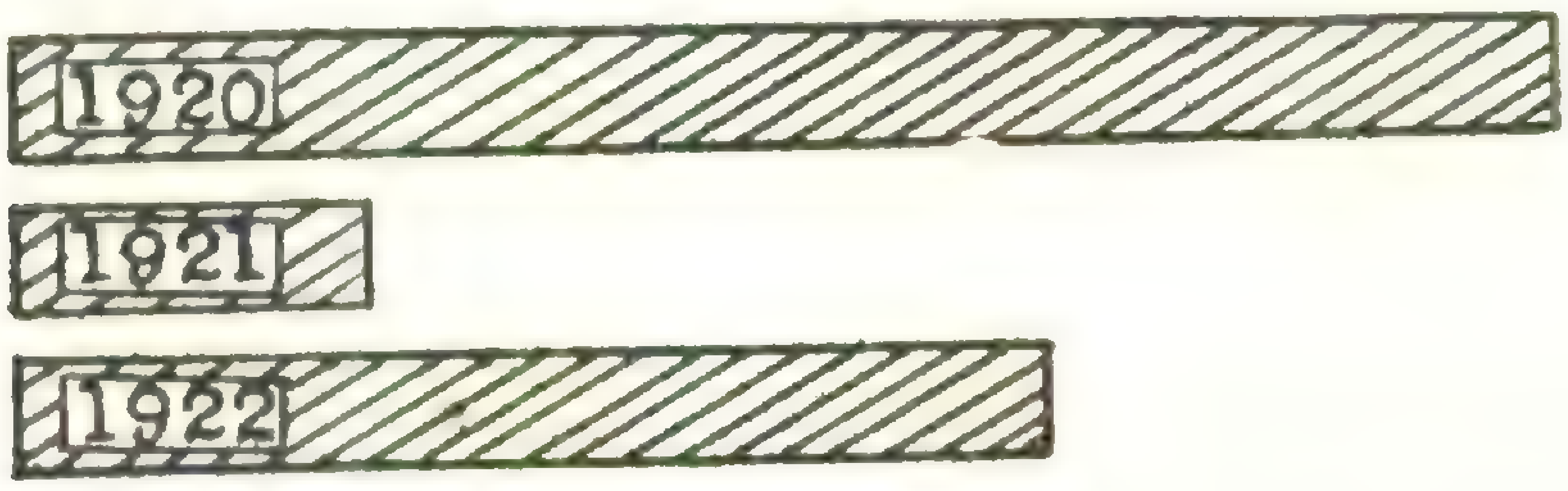

Fig. 9.

that a normal amount of rain fell during June and July. The amount decreased markedly during August and approached normality again in September. In 1920 the disease was first observed about August 1, and it spread rapidly from diseased to adjacent healthy plants during the greater part of this month, but with the continuance of dry weather in September a marked falling-off of new infections was observed. The curve for 1921 lies, for the greater part of its course, far below that representing normal rainfall. Not until September, the month having a low normal precipitation, do the two curves approach each other. In this season very few diseased plants were found and on these the disease usually involved the stem only near the ground level. In 1922, the amount of rainfall decreased from near normal in June to subnormal in July. It arose above normal in August but sank to practically nothing in September. During this season the disease appeared later and was notably less severe than in 1920, but very much more prevalent than in 1921. Figure 9 is intended to illustrate the estimated relative prevalence of this disease during these 3 summers. The monthly mean temperatures for the 3 seasons lie very close together. Apparently this factor cannot account for the prevalence of the disease noted above.

Effect of Light on Pycnidial Production

It is a matter of record that different species of fungi respond differently to the stimulus of light. Some species fail to form characteristic fruiting bodies except when exposed to light; others appear to be entirely indifferent, fruiting as characteristically in darkness as in light. Coons ('16) found that exposure to light is necessary for pycnidial production on ordinary culture media by *Plenodomus fuscomaculans*, while Harter ('13) observed that *Plenodomus destruens* developed pycnidia equally as well in darkness as in light. On the other hand, the latter worker ('13, '17) has demonstrated that light is not a necessary factor in pycnidial formation in cultures of *Diaporthe phaseolorum* and *D. batatatis*. Fewer pycnidia were formed in darkness than in light, however. A fungus believed to be *Neocosmospora vasinfecta* Smith has been found by the writer to form its perithecia in either light or darkness.

Early in the writer's experience with the soybean pod- and stem-blight fungus, *Diaporthe Sojae*, it became apparent that light is requisite for pycnidial production. Cultures kept in a dark cupboard formed mycelium abundantly but failed to develop pycnidia. When similar cultures were left standing in the laboratory where they were exposed to light, numerous pycnidia developed. This observation led the writer to test further the effect of light as a stimulating agent for pycnidial development.

Sterile soybean leaf petioles in test-tubes provided with moist cotton at the bottoms were inoculated with strain 13. The tubes were divided into two lots and kept in paste-board culture boxes in indirect light. The lid was kept on one box and left off the other. At the end of 21 days the cultures kept in the dark had developed no pycnidia, while these bodies were numerous in the cultures exposed to light. In a second test, sterile soybean stems were inoculated with strains 7 and 12. Half the tubes were kept in a covered paste-board box to exclude the light and the other half in a tall glass jar provided with a glass cover. Many pycnidia developed in cultures kept in the glass jar but none formed in those from which light was excluded. These experiments show clearly that light is a determining factor in pycnidial production by *Diaporthe Sojae*.

The writer wished next to test the effect of light of reduced intensity. Accordingly, 6 tubes containing sterile soybean petioles were inoculated with strain 7, 6 with strain 11, and 6 with strain 14. The 18 tubes were then divided into 3 lots, placing 2 cultures of each strain in each lot. Lot 1 was placed in an undarkened covered glass dish; lot 2 was kept in a covered glass dish lined with light-proof paper; and lot 3 in a similar covered glass dish lined with sufficient waxed paper to reduce the normal light intensity to approximately one-half. The 3 lots were kept on an open shelf between a north and a west window of the laboratory where they received indirect light during the day. The cultures were inoculated on June 12, and the first examination was made on August 3. Lots 1 and 3 had formed many pycnidia and were exuding pycnospores freely, while lot 2, which had been kept in total darkness, had produced no pycnidia. There was no obvious difference in the number or state of development of pycnidia in cultures kept in full and in reduced light. A light intensity of much less than half normal apparently suffices to induce pycnidial formation when cultures of *Diaporthe Sojae* are exposed continuously to it.

In order to test the relation of length of exposure to normal daylight to pycnidial production in cultures of the soybean fungus, the following experiment was carried out: On September 7, 12 tubes containing sterile soybean petioles were inoculated. The cultures were allowed to grow in complete darkness at room temperature (20–30° C.) for 7 days. They were then divided into 6 lots of 2 tubes each, and the different lots were exposed to light for periods varying from 0 to 60 hours of actual daylight and then returned to darkness. The tubes which had been exposed to 42 hours of actual daylight (72 hours of light and darkness) had already formed visible pycnidial initials when returned to darkness. The tubes were examined 10 days after returning to darkness, and all had formed many pycnidia and were exuding pycnospores. The shortest exposure was 14 hours. In these cultures, pycnidia were fewer and somewhat less well developed than in those exposed for longer periods. The tubes were returned again to darkness, and when they were reexamined on September 5, the pycnidia were about as numerous but were

obviously less well developed in the cultures exposed for 14 hours than in those subjected to light for longer periods.

A second test much like the one described above was started on October 1. The cultures were allowed to grow at room temperature (about 20° C.) for a period of 8 days in total darkness in a large covered tin box kept in a dark desk. Different lots were then exposed for periods of time varying from 6 to 36 hours of actual daylight and then returned to darkness. The tubes which had been exposed to 36 hours of daylight already bore numerous visible pycnidial initials when returned to darkness. The cultures were examined on October 29, and at this time all except the checks bore numerous pycnidia, many of which were exuding spores. The shortest exposure was 6 hours and in these cultures the pycnidia were not appreciably less numerous nor less well developed than in the tubes exposed to light for longer periods. During the period of the shortest exposure, namely, 6 hours, the sky was so cloudy that the normal light intensity was less than one-half that of normal daylight. The checks, which had never been exposed to daylight, developed no pycnidia even though they were continued in darkness until December 6. Obviously, light is essential for pycnidial production in cultures of *Diaporthe Sojae*. However, daylight of less than half normal intensity acting for a period no longer than 6 hours suffices to stimulate pycnidial development and spore formation. This is in accord with the findings of Coons ('16) who reports the formation of a small number of pycnidia in cultures of *Plenodomus fuscomaculans* exposed for 2 hours to strong indirect light.

The writer wished to try the effect of artificial light on pycnidial production. This was done in a rather crude way by the following experiment. Four cultures on sterile soybean petioles were enclosed in a white glass Mason fruit jar and 4 similar cultures were kept in a card-board box provided with a screw top to exclude light. These two lots of cultures were kept in an incubator at a distance of one foot from a 50-watt Mazda lamp. Since the lamp was being used as the heating element for the incubator, it burned intermittently. The cultures remained in the incubator at 28° C. during the entire time of the experiment, but the total time of actual illumination was less than half this period. At

the end of 3 weeks, pycnidia had formed in all the cultures in the glass can but were less numerous than when similar cultures were kept in daylight. No pycnidia developed in the cultures kept in the card-board box. A few sclerotia-like bodies did form in the moist cotton in the bottom of each tube, but these contained no spores of any sort. The response of the cultures used in this experiment to artificial light is like that observed by Coons and Levin ('20). These workers induced pycnidial formation by *Plenodomus fuscomaculans* and certain other light-sensitive fungi by subjecting cultures on agar slants to radiation emanating from two 100-watt nitrogen-filled electric bulbs. However, in the experiment reported by the writer, the light used was of less intensity and burned intermittently for only half the time.

As a result of the experiments cited above, it seems clear that, under ordinary cultural conditions, light is a necessary factor for pycnidial production by *Diaporthe Sojae.* Its intensity may be reduced to half or possibly less than half that of ordinary daylight, and the duration of illumination need not be longer than 6 hours. Moreover, electric light may be substituted for daylight. On the substratum used no consistent differences were observed between the amount of mycelium grown in cultures in light and in darkness. In this respect, the writer's observations differ from those of Coons ('16) and Harter ('17). These workers report a more abundant production of mycelium in darkness than in light. That light is only one of a number of factors operating to induce pycnidial production is evident from the writer's experience with cultures of the pod- and stem-blight fungus on other substrata. Synthetic solutions and plant decoctions solidified with agar give rise to very few, usually no, pycnidia even when kept in strong diffuse light.

Spore Germination

Under suitable conditions, pycnospores begin to germinate in 4 hours after being placed in tap water. The spores become appreciably swollen by an intake of water and put out 1 or 2 slender hyaline germ tubes. At room temperature in summer, the longest of these may reach in 18 hours a length several times that of the spore. The tubes continue to grow for about

48 hours. Germ tubes formed on the surface remain long, slender, and sparsely septate. When germination of spores immersed in the water occurs, the germ tubes soon develop septa and the cells swell and branch in an irregular manner. In addition to such requisites as proper temperature and moisture, there are certain other factors which profoundly influence germination. Much irregularity was at first experienced in attempts to germinate pycnospores. A test on one day might yield fairly high germination, whereas on the next day spores from the same culture might entirely fail to germinate. Spores from one strain might germinate well, while those from another strain of the same age and grown on the same substratum might fail to grow. Much of this irregularity was due in all probability to the use of too heavy spore suspensions in the germination tests. It has been repeatedly observed that the number of spores present in a given quantity of water has a marked influence on the percentage of germination. This fact was demonstrated in the following way: At maturity of the pycnidium, the pycnospores exude through the ostiole and cling to the tip of the beak as a small milky-colored droplet which can readily be removed by use of an inoculating needle. Single drops of tap water on depression slides were inoculated by placing one spore droplet in each drop of water. Drops of tap water on a second slide were inoculated by dipping a needle into the spore suspension on the first slide and then washing it off in the water drops on the second slide. The number of spores in the drops of the second slide was always greatly less than that in the drops on the first. These slides were kept in a moist chamber during the period of the test. In all such trials germination in the drops with the large number of spores was usually less than 1 per cent and never above 2 per cent, while, in the drops with the small number of spores, the percentage of germination seldom fell below 25, and occasionally exceeded 75. The nature of the inhibiting factor which prevents germination when an excessive number of spores are present has not been determined. It may be due to inhibition by some substance formed in the pycnidium while the spores are developing, and in that event dilution should operate to increase germination. That lack of free oxygen may be the inhibiting

condition is suggested by the fact that the spores at the margin and on the surface of the water drop are often the only ones which germinate. Very seldom does germination occur uniformly throughout a drop of spore suspension and then it is only when the drop is small or spread out as a thin film.

Pycnospores may endure severe desiccation without losing viability if allowed to dry on the beaks of the pycnidia. When first exuded, the spore balls are merely a dense suspension of pycnospores which quickly disperse by diffusion when placed in water. With loss of water the droplets become, first, doughy in consistency, and finally, as desiccation proceeds, much smaller, hard, and very dry. In this air-dry condition they do not separate readily, and considerable difficulty is experienced when one attempts to dissolve them in water. Spores in various states of dryness may be obtained from the same culture if long pieces of soybean petiole in test-tubes provided at the bottom with moist cotton are inoculated at the top. Pycnidia develop first at the upper end and exude spore droplets which, perched on the tips of the beaks, dry rapidly, while at the bottom, where the water supply is maintained by the moist cotton, pycnidia continue to exude spores for a much longer time. Germination tests have been made, using spores a few hours after they had exuded, when they were in the doughy condition and when they had become air-dry. In such a test, where spores in all 3 of the conditions cited above were taken from a 53-day-old culture and put into tap water on depression slides, the germination was approximately 50 per cent of the total for spores in all 3 conditions. In another trial with spores from a culture of the same age and strain, spores from a droplet which had recently exuded gave approximately 100 per cent germination, while air-dry spores taken from a spore droplet which had shrunk to about one-fourth its original size and had changed to the reddish brown color commonly assumed by spore balls in the air-dry condition, gave only 5 per cent germination. At another time, spores were taken from a culture 61 days old which had been kept in a wire basket and had become thoroughly dry. These spores gave 90–100 per cent germination in a mineral nutrient solution. In one instance, 25 per cent germination was obtained by use of spores

from a culture 140 days old. However, pycnospores are usually no longer viable when the culture has attained this age.

Bright diffuse daylight exercises no apparent influence on germination of pycnospores in tap water. In a number of tests in which spores from the same source were germinated in light and in darkness, approximately equal germination occurred.

Since the introduction of convenient and accurate methods for the determination of active acidity, certain workers have recorded their observations relative to the effect of the concentration of H and OH ions upon germination of fungous spores. Webb ('19, '21), using 8 different fungi and different types of media, found that the amount and range of germination as influenced by H-ion concentration varied with the organism and the medium. The greatest number of fungi employed exhibited maximum germination between P_H 3.0 and 4.0, the percentage of germination decreasing rapidly at higher, and less rapidly at lower, concentrations of H ions. *Fusarium* showed marked tolerance, and *Colletotrichum Gossypii* favorable response, to alkaline reaction of the culture medium. Hursh ('22), working with urediniospores of 2 biologic forms of *Puccinia graminis Tritici,* found marked differences in the germination quantities at the same H-ion concentrations over the greater part of the range where germination occurred. These differences were much greater at temperatures of 10 and 30° C. than at 20° C. The range indicated was approximately P_H 2.5–8.0, and best germination occurred between P_H 4.5 and P_H 6–7. Maneval ('22) found that teliospores of *Puccinia Helianthi* will germinate in solutions having a range of H-ion concentrations represented by the P_H values 3.5–8.4. However, the limits for very good germination and good sporidial production were approximately P_H 4.6–6.5.

The writer tested the effect of H-ion concentration on the germination of pycnospores of *Diaporthe Sojae* in a synthetic solution consisting of inorganic salts dissolved in distilled water in the following volume molecular proportions: M/5 KNO_3, M/20 KH_2PO_4, and M/100 $MgSO_4$. Three drops of M/1000 $FePO_4$ were added to each 25 cc. of solution. The desired H-ion concentrations were obtained by adding previously determined

amounts of 0.5766N H_3PO_4 or 0.1005N KOH to 25 cc. of the nutrient solution. Approximately 4 cc. of each adjusted solution was placed in a clean test-tube, and to each of these was added 0.1 cc. of a heavy spore suspension in distilled water. The addition of this amount of spore suspension produced no change of reaction between P_H 3.1 and 8.3 inclusive, and only very inconsiderable change outside these bounds. Germinations were made in hanging drops, or Van Tieghem cells, and due precautions

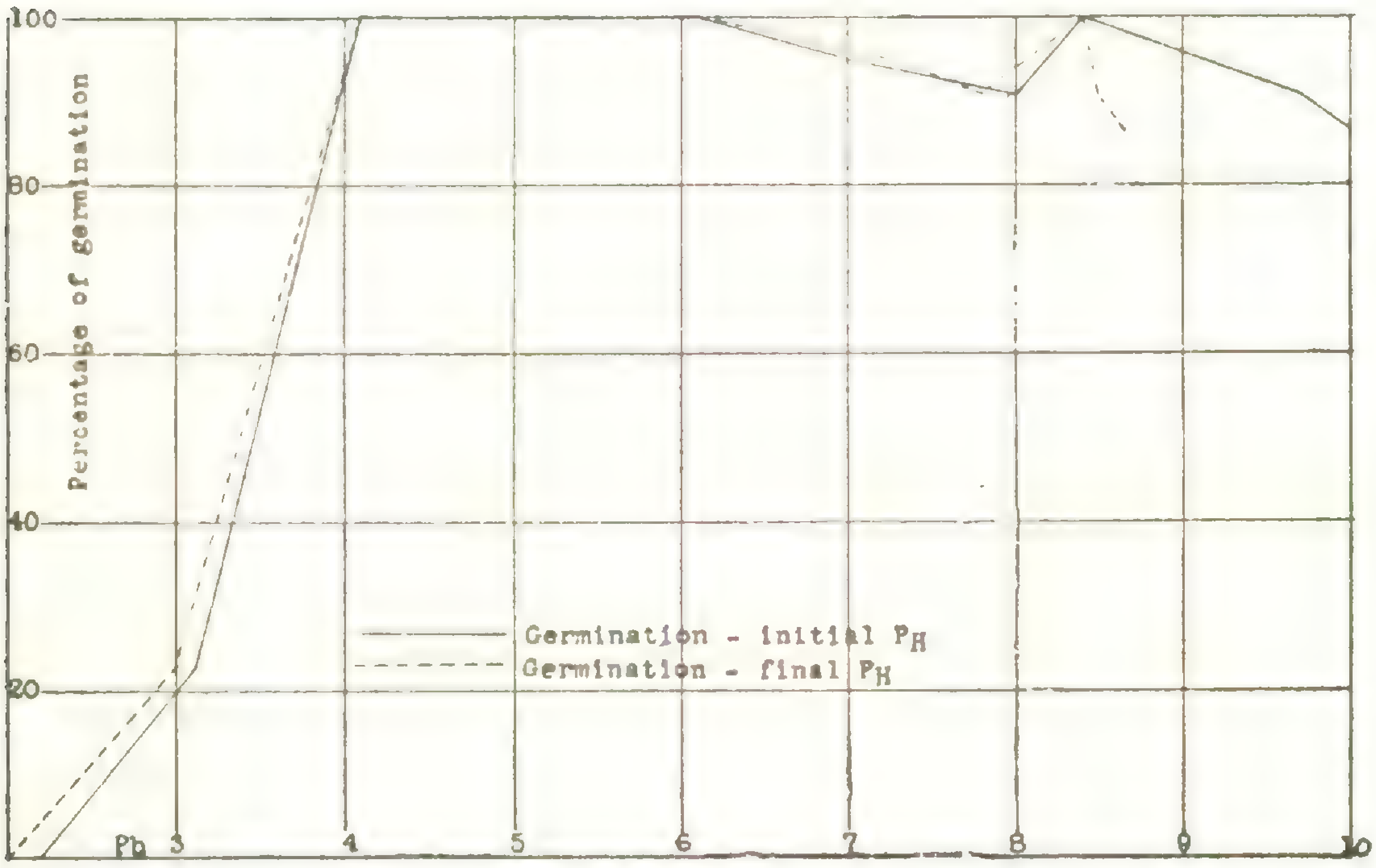

Fig. 10.

were observed relative to the cleanliness of the slides, rings, and cover-glasses. By use of a platinum loop small drops of the spore suspension were transferred to cover slips, and these were in turn sealed over the glass rings by means of petrolatum. The platinum loop was flamed and cooled before being used on each solution. Several drops of the appropriate solution were placed in the bottom of each cell. Duplicate cells were prepared for each solution of a different P_H value. These were maintained at a temperature of 25° C. during the germination period. At the end of 22 hours, the amount of germination was determined by taking the average of the counts of 10 different fields, a total of 200–300 spores being involved in each average.

The results are graphically presented in fig. 10. The solid line correlates germination with the P_H value of the spore suspension at the beginning of the test, while the broken curve expresses the relation between the P_H value of the uninoculated solution as found 40 hours later. No germination occurred at the hydrogen-ion concentration represented by P_H 2.2. At P_H 3.1, a germination of 22.3 per cent took place, but the germ tubes were very short, most of them not as long as the spores. One hundred per cent of the spores germinated, producing tubes several times as long as the spore at P_H 4.1, 5.3, and 6.1. The percentage of germination decreases slightly at P_H 7 and 8, but increases again to a secondary maximum at P_H 8.4 and falls again in the more alkaline solutions. Between P_H 4.1 and 8.4 the germ tubes are of approximately the same uniform length, but beginning at P_H 9.7 they become shorter and at P_H 10.0 are markedly shorter than at 8.4 but not yet so short as at P_H 3. A germination amounting to 21.6 per cent occurred in distilled water having a P_H value of 5.1 but here the germ tubes were very short. Thus, it is seen that much better germination may be obtained in the mineral nutrient used than in distilled water, and that very good germination occurs over a wide range of hydrogen-ion concentration.

Effect of the Reaction of the Substratum on the Growth of Mycelium

That the degree of acidity or alkalinity of the medium profoundly influences growth of fungi is a fact attested by numerous recorded observations. In general, growth has been observed to be more marked on the acid side of neutrality, comparatively few of the species studied producing equally favorable or better growth in alkaline media. Omitting all records relating exclusively to bacteria and all not definitely stating the reaction in terms of active acidity, a partial account of the observations of other workers bearing on the relation of growth to hydrogen-ion concentration is given below:

Meacham ('18), growing *Lenzites sepiaria, Fomes roseus, Coniophora cerebella,* and *Merulius lacrymans* on synthetic and

malt extract media adjusted to different H-ion concentrations, found that although distinct variations were present, the 4 fungi responded in much the same way. The limiting P_H value appeared to be near 1.7, though very little growth occurred below P_H 2.2. Maximum growth was obtained at about P_H 3.

Zeller, Schmitz, and Duggar ('19) grew 12 species of wood-rotting fungi on 6 kinds of culture solutions. The data obtained show that vigor of growth, limiting H-ion concentration, and direction of shift of the P_H during growth depended both upon the fungus and the culture medium. These workers thought it inadvisable to formulate any general statement purporting to express the relation between hydrogen-ion concentration of the culture media and growth of wood-destroying fungi as a group.

Armstrong ('21) studied sulphur nutrition of fungi on a medium containing inorganic salts and sucrose. Comparing the average of several different determinations, he obtained better growth of *Aspergillus niger*, *Penicillium cyclopium*, and *Botrytis cinerea* at P_H 4.1 than at 5.5. When $Na_2S_2O_3$ was substituted for $MgSO_4$, *A. niger* and *B. cinerea* grew best at P_H 5.9 and *P. cyclopium* at P_H 4.2, within a range of 4.2 –7.1 Changes of H-ion concentration during growth varied with the organism and the culture solution.

Karrer ('21), using Czapek's solution in which soluble starch had been substituted for most of the sucrose, found no growth of *Fusarium* at P_H2 and approximately equal growth between P_H 3 and 9.2. *Colletotrichum Gossypii* exhibited fair growth from P_H 3–4.5 to beyond 9.2. Both fungi caused a shift of reaction of the culture solution, the change, except for the most alkaline cultures, being in the direction of increased alkalinity.

Kirby ('22) found that *Ophiobolus cariceti* requires a condition of alkalinity for optimum growth on agar media. On corn meal agar growth began at P_H 4.5, reached a maximum at 8.1, and was still good at 9.2. On potato agar growth began at P_H 3.2 and attained a maximum at 9. For *Fusarium moniliforme* on corn meal agar, the range of growth was found to be greater than P_H 3.2–9.2, the maximum occurring near 8.2.

Hopkins ('22) found that the growth of *Colletotrichum lindemuthianum* on potato dextrose agar adjusted to different H-ion

concentrations by the addition of increasing amounts of lactic acid produced in 10 days colonies of greater diameter on media having a P_H value of 4.5 than on the same medium with P_H 3.8, 4, and 7.4, the 3 other values used. On the other hand, conidial production was 25 to 30 times greater at P_H 3.8 than at 4.

MacInnes ('22) observed that a strain of *Fusarium* sp. isolated from scabby wheat grew on a modified Czapek's solution at P_H values ranging from 3 to 11.7. No determinations were recorded for H-ion concentrations immediately above these values.

The writer has studied the growth of *Diaporthe Sojae* on a nutrient solution containing inorganic salts and dextrose in the following volume molecular concentrations: M/3.636 dextrose, M/5 KNO_3, M/20 KH_2PO_4, and M/100 $MgSO_4$. Three drops M/1000 of $FePO_4$ were added for each 25 cc. of solution. The dextrose was of the grade designated "difco standardized" and the inorganic salts were of Merck's "highest purity" grade except the KH_2PO_4, the grade of which was "C.P." The nutrients were dissolved in water which had been redistilled from glass. After sterilization the solution was adjusted to the desired P_H values by adding previously determined amounts of sterile 0.5766 N H_3PO_4 of 0.5031 N KOH. The quantities added caused only inconsequential change in the molar concentrations of the nutrients and did not alter total NO_3 and dextrose. The P_H values were determined by the colorimetric method of Clark and Lubs ('17). The cultures were grown in 100-cc. Pyrex flasks which had been treated with cleaning mixture and thoroughly rinsed in tap and distilled water. Twenty-five cc. of solution were placed in each flask. Four days after the solutions had been prepared, inoculations were made by introducing into each flask a single pycnidium together with a small amount of mycelium from a young culture on soybean petiole. The cultures were kept at 25° C. in a dark incubator during the period of growth. By use of a suction filter the mats were collected on a filter-paper, the dry weight of which had been previously determined. The mats were dried for 3 days in an electric oven kept at 100–105° C., cooled in a H_2SO_4 desiccator for a uniform period, and weighed. After the filtrate from each mat had been made up to 50 cc. by the addition of distilled water, the hydrogen-

ion concentration, total acidity, and amount of sugar were determined. Total acidity was determined by titrating a 10-cc. aliquot of the 50 cc. of filtrate with 0.10055 N KOH, using phenolphthalein as an indicator. The micro method of Shaffer and Hartmann ('21) was employed for the sugar determination. Determinations were made on each member of the duplicate cultures.

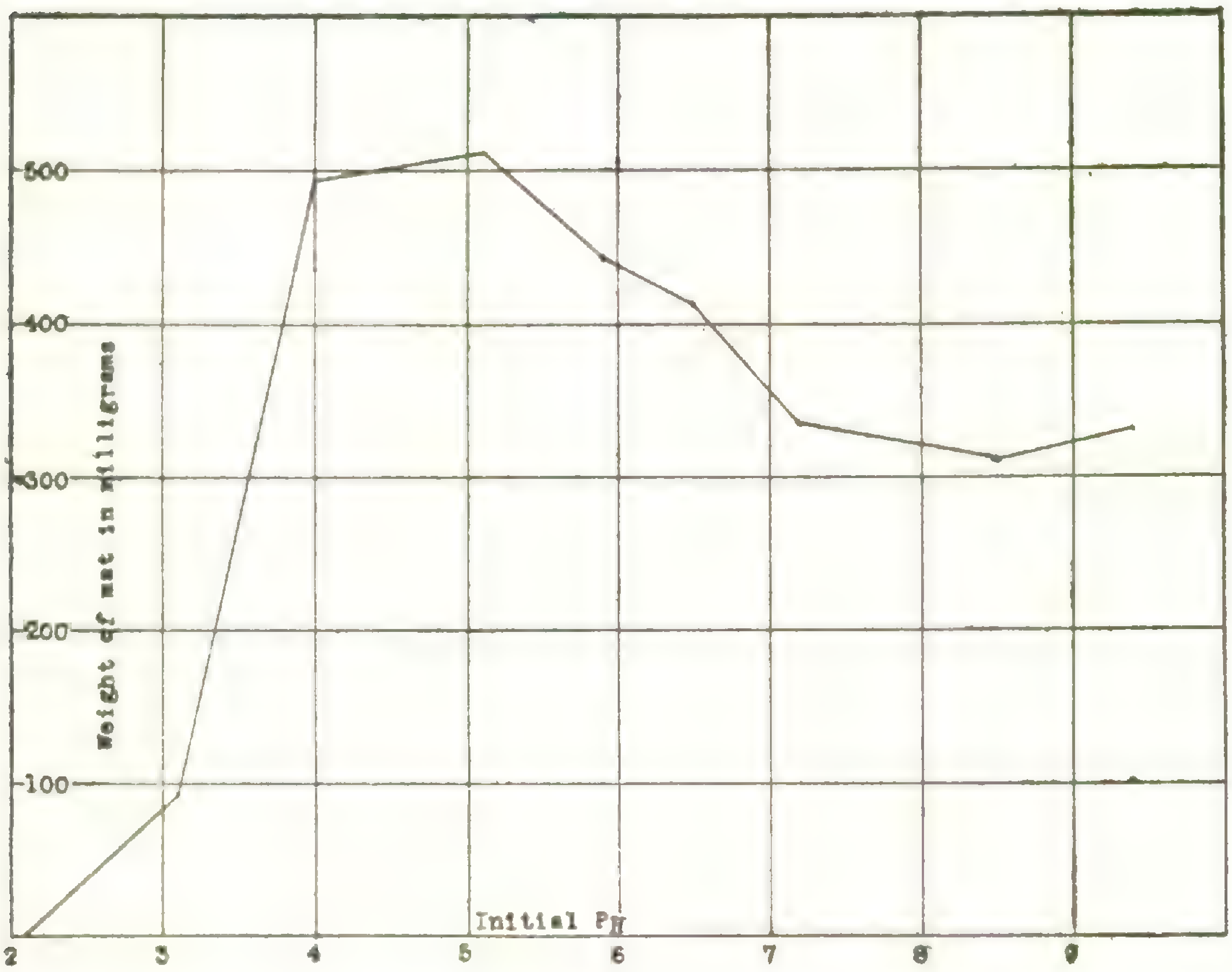

Fig. 11.

Experiment I.—In this test, cultures were started at the initial H-ion concentrations represented by P_H 1.8, 2.1, 3.1, 4.0, 5.1, 5.9, 6.5, 7.2, 8.5, and 9.4, and were allowed to grow for a period of 18 days. At the end of this time, best growth, as indicated by dry weight of mat, was found to have occurred in the cultures started at P_H 4 and 5.1. No growth took place at P_H 1.8 and 2.1, and growth was less at P_H 3.1 than at any higher P_H exponent. A second minimum occurred at P_H 8.5, but there was very little difference between the amounts at P_H 7.2, 8.5, and 9.4. Growth was accompanied by a shifting of the reaction of the culture

solution. All cultures initially acid became less so, while all initially alkaline became slightly acid. The P_H value of the uninoculated controls remained stationary with the exception that those of P_H 8.5 and 9.4 shifted to 7.9 and 8.4 respectively. A graphic representation of the correlation between growth and the initial P_H values of the culture solution is presented in fig. 11.

A consideration of the results of the Experiment I made it seem desirable to follow more closely certain changes which occur in the culture solution during growth. It did not seem that the small amount of growth in the cultures having an initial P_H of 3.1 and 8.5 as determined at the end of 18 days represented the amount possible, considering that H-ion concentration shifts to a more favorable region during growth and that the same amount of sugar was present initially in all the cultures. Also, it appeared that a knowledge of the changes of both active and total acidity and of utilization of sugar during growth might be of interest. Accordingly, a second experiment was planned involving a larger number of cultures, thus making frequent determinations possible.

Experiment II.—Nine series consisting of 4–15 flasks were prepared. All the flasks of a series were started at the same H-ion concentration and a separate series was prepared for each initial P_H value selected. All the cultures were inoculated at the same time and examinations were made at intervals of 3–7 days on 2 cultures from each series. Dry weight of mat, amount of sugar, H-ion concentration, and titrable acidity were determined at each examination. Table I embodies the data obtained, and the curves of fig. 12 indicate such relation as may exist between certain factors and processes resident in the cultures involved. The curves (fig. 12) expressing the relation of time to growth, starting at different H-ion concentrations, are very much alike for series 3–8 inclusive. They rise rapidly to a maximum, or thereabout, then flatten, and decline slightly. The curve for series 2 is at first concave and indicates slower growth at the start, but it eventually rises to a height nearly equal that attained by the curves for the most favorable solutions. Growth in series 10 was very irregular, some flasks starting much earlier than others. The maximum growth in this series was less than that in any other. In series 1, started at P_H 2.5, growth did not

TABLE I

GROWTH IN NUTRIENT SOLUTION, EXPERIMENT II*

Series No.	Acid or alkali added to 25 cc. of nutrient sol.		Initial P_H		Growth period in days							Control
	0.576 N H_3PO_4	0.5031 N KOH 7			6	9	13	16	20	27	34	
2	0.92		3.0	Wt. of mat	18	19		147	297	494	453	3.0
				Wt. of sugar	47.5	45.5		33.2	20	0	0	
				Final P_H	3.1	3.2		5.5	5.8	7.0	7.2	
				KOH to neutralize	16.25	16.67		14.17	13.12	5.5	3.0	
3	0	0	4.0	Wt. of mat		188		476	514	462	453	4.0
				Wt. of sugar		31		8.0	0	0	0	
				Final P_H		5.7		6.6	7.0	7.8	8.2	
				KOH to neutralize		10.37		6.5	5.75	1.25	1.12	
4		0.14	5.5	Wt. of mat		203		472	490	446	428	5.5
				Wt. of sugar		31		1.3	0	0	0	
				Final P_H		6.1		6.6	7.1	8.4	8.5	
				KOH to neutralize		9.87		6.5	3.5	1.12	0.75	
5		0.5	6.1	Wt. of mat	89	207	481	482	483	505	458	6.1
				Wt. of sugar	41.1	32.4	1.6	3.3	0	0	0	
				Final P_H	6.3	6.2	6.8	6.7	7.8	8.3	8.5	
				KOH to neutralize	10.75	9.25	5.62	6.5	1.62	1.0	0.82	

*The values presented represent the averages of 2 cultures. Dry weight of mat and weight of unused sugar per cc. of culture solution are given in milligrams. The P_H values represent the averages of the H-ion concentrations for duplicate cultures. KOH shows the number of cc. of 0.1005 N KOH to neutralize the filtrate.

TABLE I—*Continued*

Series No.	Acid or alkali added to 25 cc. of nutrient sol.		Initial P_H		Growth period in days							Control
	0.576 N H_3PO_4	0.5031 N KOH 7			6	9	13	16	20	27	34	
6		1.7	7.0	Wt. of mat		153		394	429	395	334	6.9
				Wt. of sugar		34.6		10.1	0	0	0	
				Final P_H		6.7		6.5	6.7	7.5	7.3	
				KOH to neutralize		6.5		6.92	5.37	2.25	2.5	
7		2.4	7.6	Wt. of mat	114	203	458	460†	427	441	413	
				Wt. of sugar	36.5	22	0.4	0†	0	0	0	
				Final P_H	7.3	6.7	7.6	7.7†	7.0	7.2	9.0	
				KOH to neutralize	3.0	5.67	1.8	2.0†	3.62	2.7	0	
8		3.2	8.7	Wt. of mat		190		461	501†	417	373	8.7
				Wt. of sugar		26		0	0†	0	0	
				Final P_H		7.6		8.7	8.5†	9.0	9.9	
				KOH to neutralize		1.75		0.69	0.5†	0.55‡	1.37‡	
9		3.6	8.9	Wt. of mat		221		226	329	411	410	8.9
				Wt. of sugar		19.8		15.1	9.0	1.9	0.7	
				Final P_H		7.7		7.1	6.6	6.7	7.3	
				KOH to neutralize		1.37		3.25	6.25	5.5	2.0	

† One culture only.
‡ N 0.1005 HCl.

become apparent until about the sixteenth day. It first appeared as a few hyphae clinging to the sides of the flasks and increased so slowly that at the end of 34 days the amount was judged to be not more than 20 to 30 mgs. dry weight, and at the end of 20 days more little, if any more, growth occurred. The fungus is apparently able to live in contact with a solution as

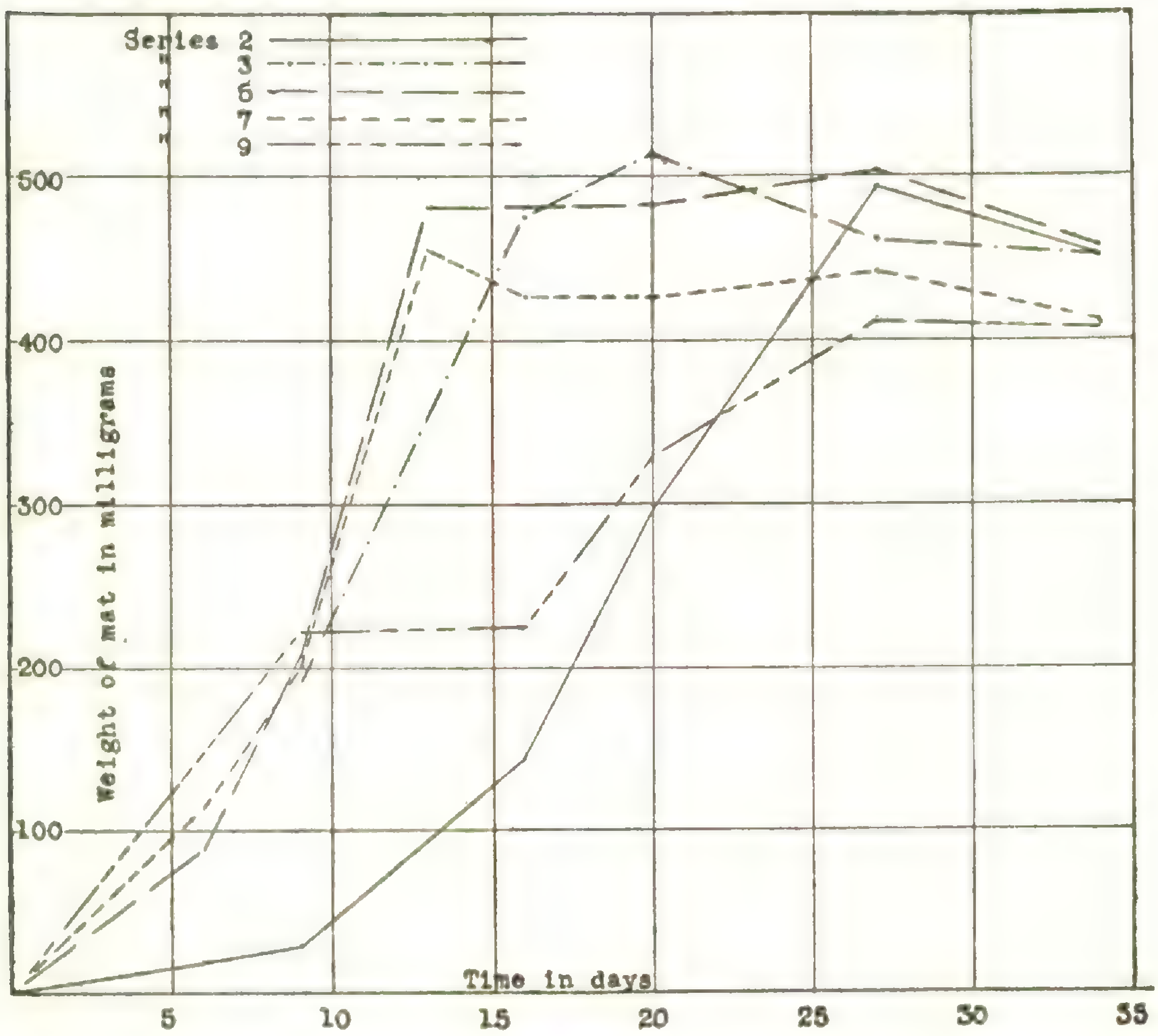

Fig. 12.

acid as P_H 2.5, but makes very slow growth; while at P_H 3 growth is slow until the fungus is able to bring about a more favorable reaction, whereupon growth increases rapidly.

All cultures on the acid side of P_H 7 steadily became less acid as growth advanced, reaching approximately P_H 7 by the time maximum growth was attained, and becoming distinctly alkaline thereafter. Cultures started at P_H 7 or above first became less alkaline, then reversed the direction of change and became more

alkaline than at the start. Exclusive of No. 10, in which growth was very irregular, these series recovered their original P_H value at the approximate time of attainment of maximum growth.

Changes in titrable acidity in general follow change in hydrogenion concentration. The P_H value of the control flask for each series remained stationary until the end of the experiment.

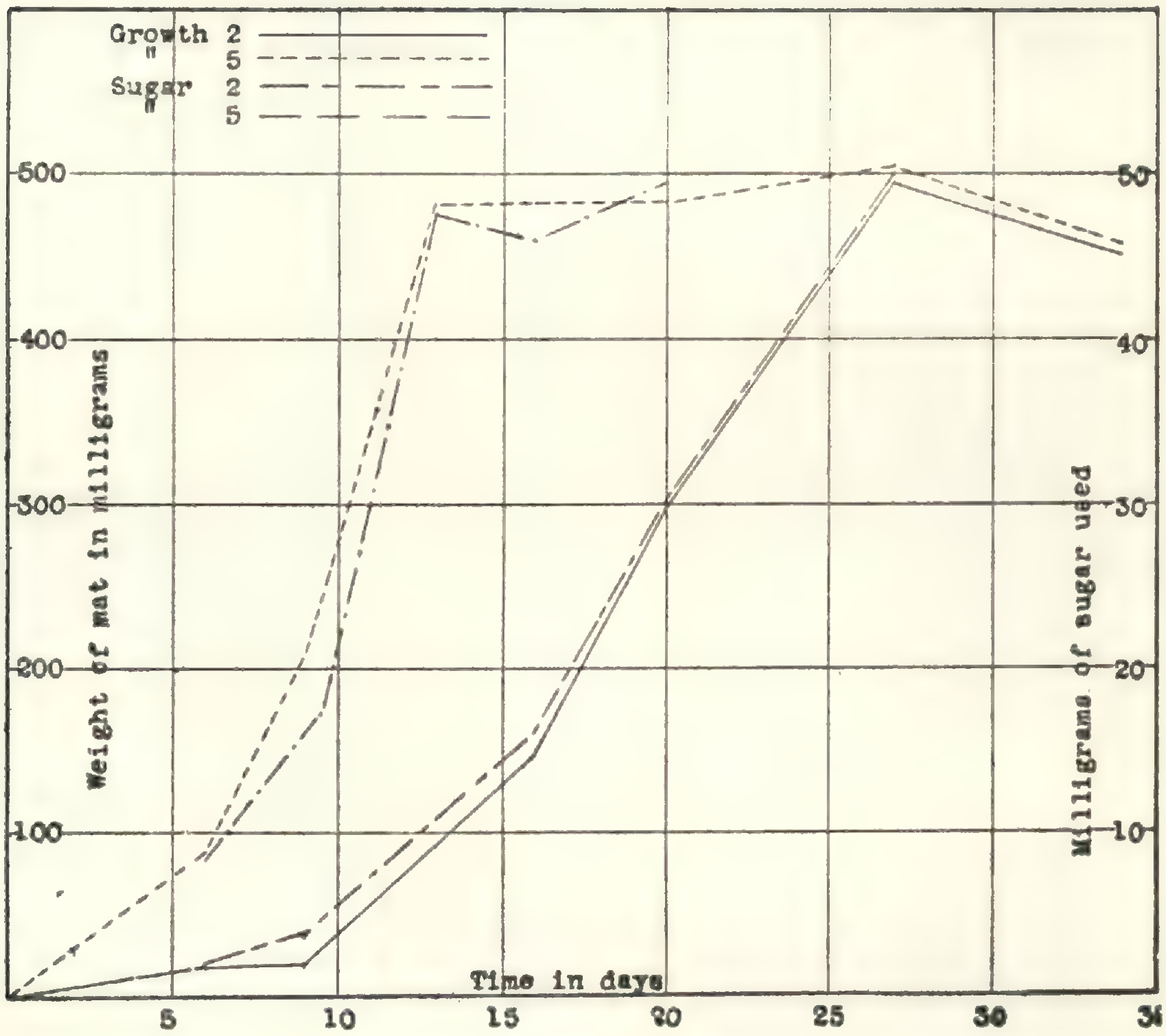

Fig. 13.

Decrease of sugar in the culture solution was closely correlated with increase of dry weight of mat. In fig. 13 the curves representing disappearance of sugar very closely follow those representing increase of growth. On the dates when maximum dry weight of mat was found, sugar had entirely disappeared from the culture solution.

In a third experiment made for the purpose of testing the effect of hydrogen-ion concentration on pycnidial production, data were obtained relative to the growth of *Diaporthe Sojae* on solid media.

The fungus was grown on 1½ per cent agar containing M/50 dextrose, M/50 KNO_3, M/100 KH_2PO_4, M/500 $MgSO_4$, and 5 drops of M/1000 $FePO_4$ per 1000 cc. of solution. The medium was sterilized in 100-cc. quantities and the reaction was then adjusted by the addition of appropriate quantities of sterile 0.5 N H_3PO_4 or 0.5 N KOH. After it had stood for several days, it was then poured into large petri dishes and the P_H values were determined. The petri dishes were inoculated by placing a

TABLE II

GROWTH OF MYCELIUM ON AN AGAR MEDIUM OF DIFFERENT INITIAL H-ION CONCENTRATIONS

Culture No.	P value	Av. diam. of colonies in mm.			Remarks
		7 da.	10 da.	Gain	
1	2.9	20	32	12	Mat floccose
2	3.5	35	61	26	Mat floccose
3	5.2	62	92		Mat less floccose
4	6.0	67	92		Mat less floccose
5	6.8	58	92		Mat less floccose
6	7.2	60	92		Mat less floccose
7	8.2	51	92		Mat less floccose

single pycnidium at the center of each and the cultures were then placed in light in a glass incubator maintained at 25–30° C. As shown in table II, the diameters of the colonies were measured at 2 different times. Under such conditions, growth was found to be most rapid when started at P_H 6 and to fall off at either end of the range used. The colonies in the plates having an initial P_H of 2.9 and 3.5 grew comparatively slowly but eventually reached the margins of the plates.

CONTROL

No experimental work has yet been done on the control of this disease. However, the life history of the causal organism, its relation to its host plant, and the course of the disease in the field suggest certain measures by which losses may be minimized, if not entirely prevented. Halsted reported encouraging results from spraying with Bordeaux mixture for control of pod blight of lima beans, and Harter ('17) recommends that spraying begin

when the plants are 1 or 2 feet high and be repeated often enough to keep the foliage covered. Because of the manifest similarity between pod blight of lima bean and pod and stem blight of soybean, it seems that spraying should reduce the losses from the latter disease sufficiently to render the operation profitable. Because of the difficulties encountered in spraying plants with dense foliage, such as is found on soybeans, it is possible that dusting with Bordeaux or sulphur might give more satisfactory control because of better penetration of dense foliage by dust than by spray. The coincidence of the time of most abundant infections and the summer period of heavy rainfall is a factor of considerable importance bearing on any spraying or dusting schedule that may be proposed. The protective material must be something that will stick to stems, pods, and leaves during rainy weather. However, the hairy nature of pods and stems would assist in maintaining the protective film on the plant parts.

Diseased seeds have yielded isolations of the causal fungus in April and May following the season in which they were grown. In one case the fungus was isolated from seed obtained from diseased plants 17½ months after harvest. These plants were kept in the laboratory during the interval between harvest and the time of making the isolation, and the seeds were disinfected in alcoholic mercuric chloride and germinated on moist sterile blotting-paper in large test-tubes. Thus it seems probable that the fungus may remain viable even to the second planting season after harvest.

The fact that the organism causing this disease penetrates the seed-coats and passes the winter as an internal mycelium renders ineffective treatment of seeds by ordinary surface disinfectants. Certain workers have shown, however, that certain seed-borne plant parasites lose their vitality more rapidly than the seeds carrying them. Barre ('12) found that the cotton anthracnose organism remains alive in the seed until the second season, but that 3-year-old seed produces disease-free plants when planted in the field. Rapp ('19), working with bacterial blight of beans, has found that 3-year-old beans produce disease-free plants. Thus it seems probable that, in the case of pod and stem blight

of soybean, seed-borne infection may be eliminated naturally with increase in age of the diseased seed.

Inasmuch as this fungus is able to maintain its viability during the winter and produce pycnospores on old stems lying in the field in the spring, a very evident and important precaution in control is removal of diseased plant parts after harvest. Doubtless, this can be accomplished most economically by plowing them under deeply. Rotation is suggested as a further measure of precaution.

Summary

(1) A disease herein called pod and stem blight of soybean has been studied and described.

(2) The disease is not known to be widely distributed, having been found to date in only 3 localities, all of which are in North Carolina.

(3) The disease occurs on pods, stems, and infrequently on leaves. It causes a premature death of plants, a failure of young ovules to develop, and a moulding and decay of seeds in later stages of development.

(4) The presence of the causal organism is manifested by the appearance of pycnidia more or less generally distributed over the stems and pods. Perithecia have never been found in the field, but have developed in cultures of 2 strains isolated from diseased pods. By pure culture methods the genetical relation of the perithecial and pycnidial stages of the causal organism has been demonstrated.

(5) The causal organism is believed to have been hitherto undescribed. Its characters place it in the genus *Diaporthe* Nitschke, and in reference to its host it has been assigned the name *Diaporthe Sojae*.

(6) The causal organism has been isolated from stems, pods, and seeds. It has been observed to cause the death of very young seedlings by growing from the seed-coat to the hypocotyl and causing it to decay. Successful inoculations have been made in the field and greenhouse and the organism has been recovered from plants diseased as the result of artificial inoculation.

(7) The causal organism overwinters on diseased stems and in diseased seed. Pycnospores are produced in abundance in the spring on diseased stems which have lain in the field over winter. Diseased seed have yielded isolations of the causal fungus in April and May following the season in which they were grown.

(8) Black Eyebrow is apparently more susceptible than any of the other varieties observed.

(9) Infection and dissemination of this disease during the growing season is strongly dependent on relatively high humidity, the disease being markedly more abundant during rainy than during dry summers.

(10) The fungus has been grown in culture on a variety of substrata. In general, pycnidia are not formed numerously on agar media, cooked rice, or potato plugs. They are produced in large numbers on sterile soybean stems and petioles and on stems of *Melilotus alba*. Perfect strains produce perithecia on all media on which pycnidia are formed abundantly.

(11) In a nutrient solution containing inorganic salts and glucose, pycnospores germinate over a wide range of hydrogen-ion concentrations. The lower limit for germination lies between P_H 2.2 and 3.0 and the upper limit beyond 8.6. The range P_H 4.1–6.1 is apparently optimum for germination.

(12) Certain changes occurring during growth of mycelium in a nutrient solution containing inorganic salts and glucose have been followed. Growth was nil at P_H 1.8 and 2.2, and extremely slow at 2.5. Growth starting at P_H 3.1 was much slower than at P_H 4.0, but dry weight of mat produced at the former value nearly equalled that attained in the latter. Maximum growth occurred in the series started at P_H 4.0 Good growth occurred in the series started at P_H 8.9 but was slower a id notably less in amount than in the series started at 8.7. Sugar disappeared from the solution concomitantly as the weight of mat increased. All sugar had disappeared from the culture solution when maximum growth had been attained. Changes in reaction of the culture solution occurred during growth. Cultures initially acid steadily became less so, and cultures originally alkaline first became less alkaline, then reversed the direction of change and became more alkaline than at the start.

(13) Light is essential to pycnidial development, no pycnidia forming in cultures kept in total darkness during their entire growth period. No longer than 6 hours' exposure to daylight whose intensity has been reduced to one-half that of bright diffuse light is required to bring about pycnidial production in cultures on favorable media. Electric light is effective in inducing pycnidial production.

(14) For the control of this disease, the practice of such sanitary measures as the removal of diseased plants, the use of disease-free seed, and crop rotation are to be recommended.

Acknowledgments.—The writer wishes to express his thanks to Dr. B. M. Duggar for kindly direction and criticism in the latter part of this work, and to acknowledge his appreciation of the helpful advice of Dr. F. A. Wolf, of the department of botany and plant pathology of the North Carolina Agricultural Experiment Station, under whose direction this work was begun. Acknowledgments are also due Dr. George T. Moore for the privileges and facilities of the Missouri Botanical Garden. Mr. A. F. Camp kindly made certain of the photographs.

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Explanation of Plate

PLATE 9

All drawings made with the aid of the camera lucida.

Fig. 1. Section of the wall of a pod showing mycelium in the cells.

Fig. 2. Section of a stem showing mycelium in cells of a vascular bundle.

Fig. 3. Mycelium in the indurated seed-coat.

Figs. 4, 5, and 6. Hyphae penetrating the walls of tracheids.

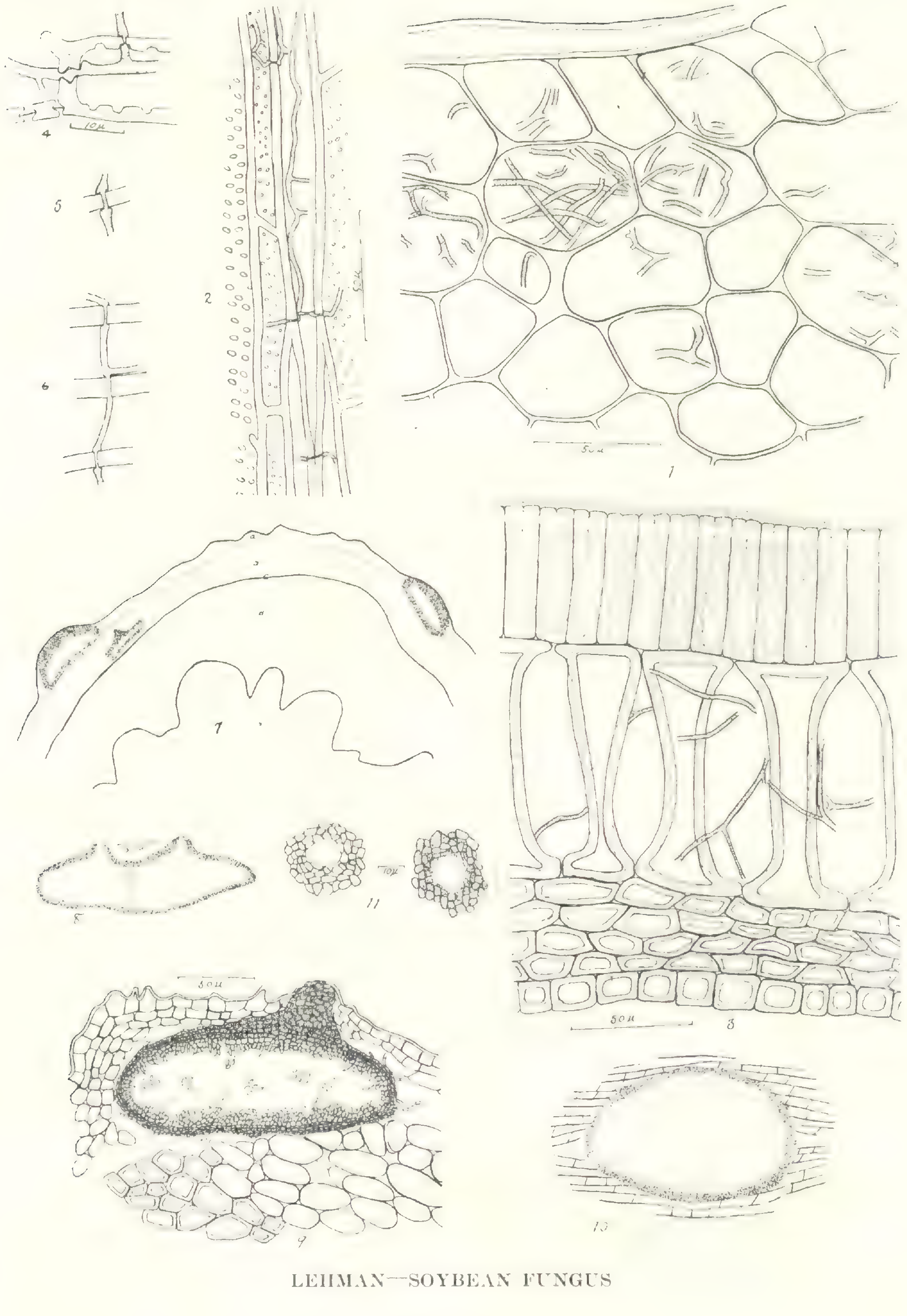
Fig. 7. Cross-section of a stem showing position of the pycnidia. a, portion of the cortex composed of cells with thin walls; b, portion of cortex composed largely of cells with thick walls; c, position of the endodermis.

Fig. 8. Pycnidium with two chambers.

Fig. 9. Cross-section of a stem showing a vertical section of a pycnidium.

Fig. 10. Cross-section of a pycnidium showing the oval outline.

Fig. 11. Sections of a beak of a pycnidium.



LEHMAN—SOYBEAN FUNGUS

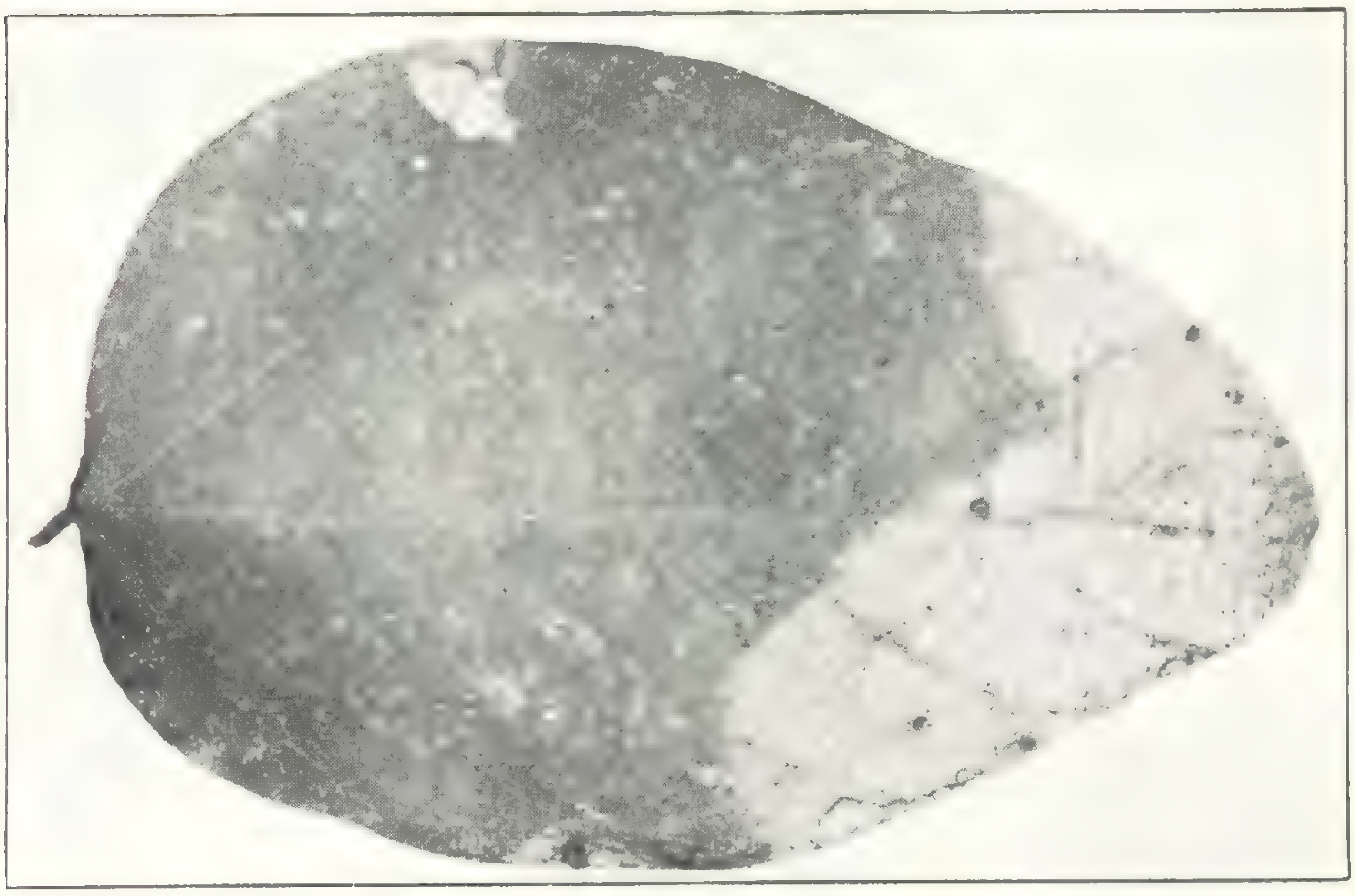
Explanation of Plate

PLATE 10

Fig. 1. Pods which became diseased as the result of inoculation with a spore suspension of pycnospores. Numerous black pycnidia are scattered over the surface of the pods.

Fig. 2. Leaf of soybean plant bearing numerous pycnidia on the diseased areas.

1



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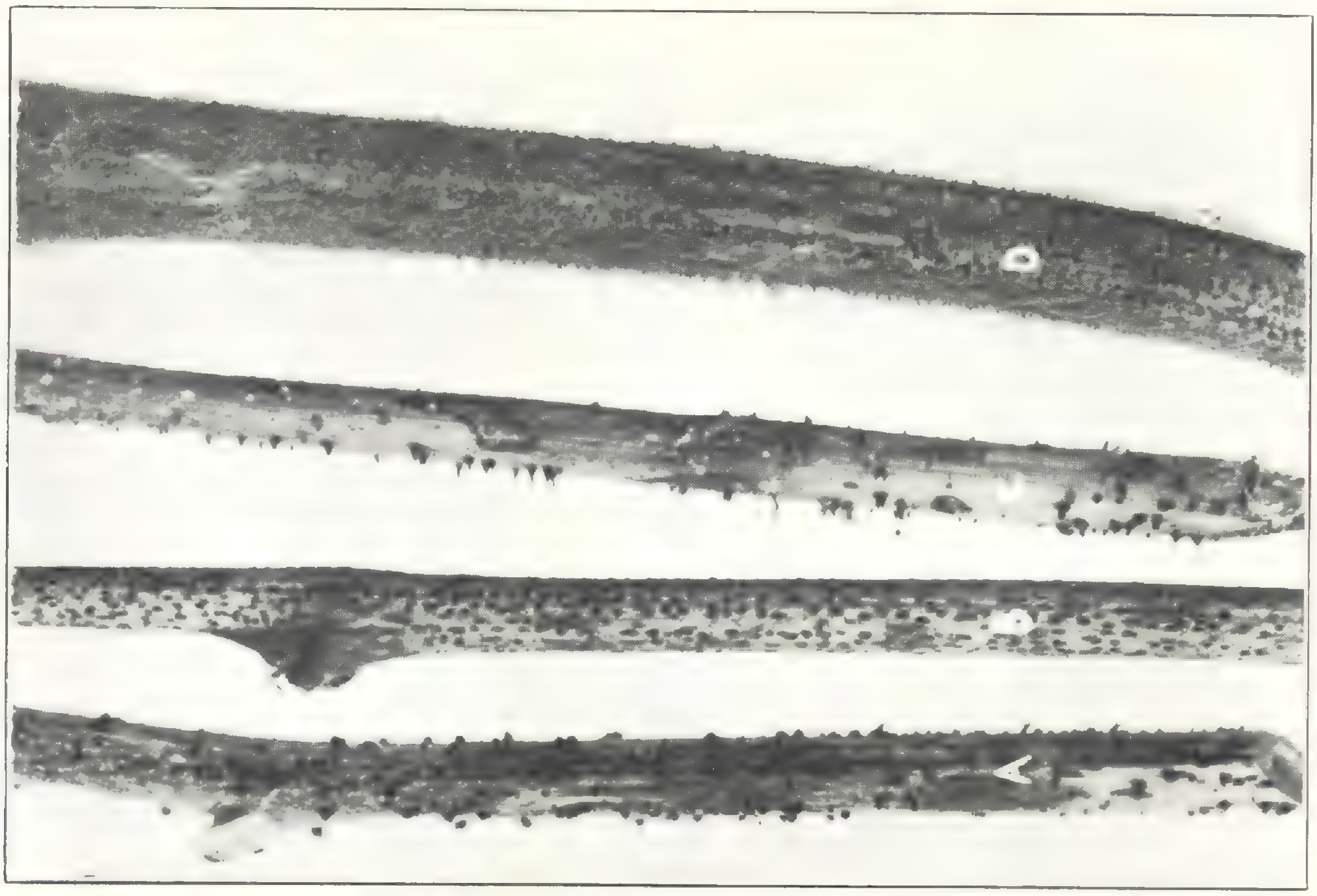
LEHMAN—SOYBEAN FUNGUS

Explanation of Plate

PLATE 11

Fig. 1. Pycnidia of *Diaporthe Sojae:* A, autoclaved stem of *Melilotus alba*; B, stem of soybean plant grown in the greenhouse and artificially inoculated; C, autoclaved petiole of a soybean leaf (Note the long beaks that often develop in culture); D, autoclaved stem of a soybean plant.

Fig. 2. Perithecia of *Diaporthe Sojae* formed in cultures on autoclaved petioles of soybean leaves. Note the tendency to form in clusters and the long curved and crooked beaks. Photograph by Mr. A. F. Camp.



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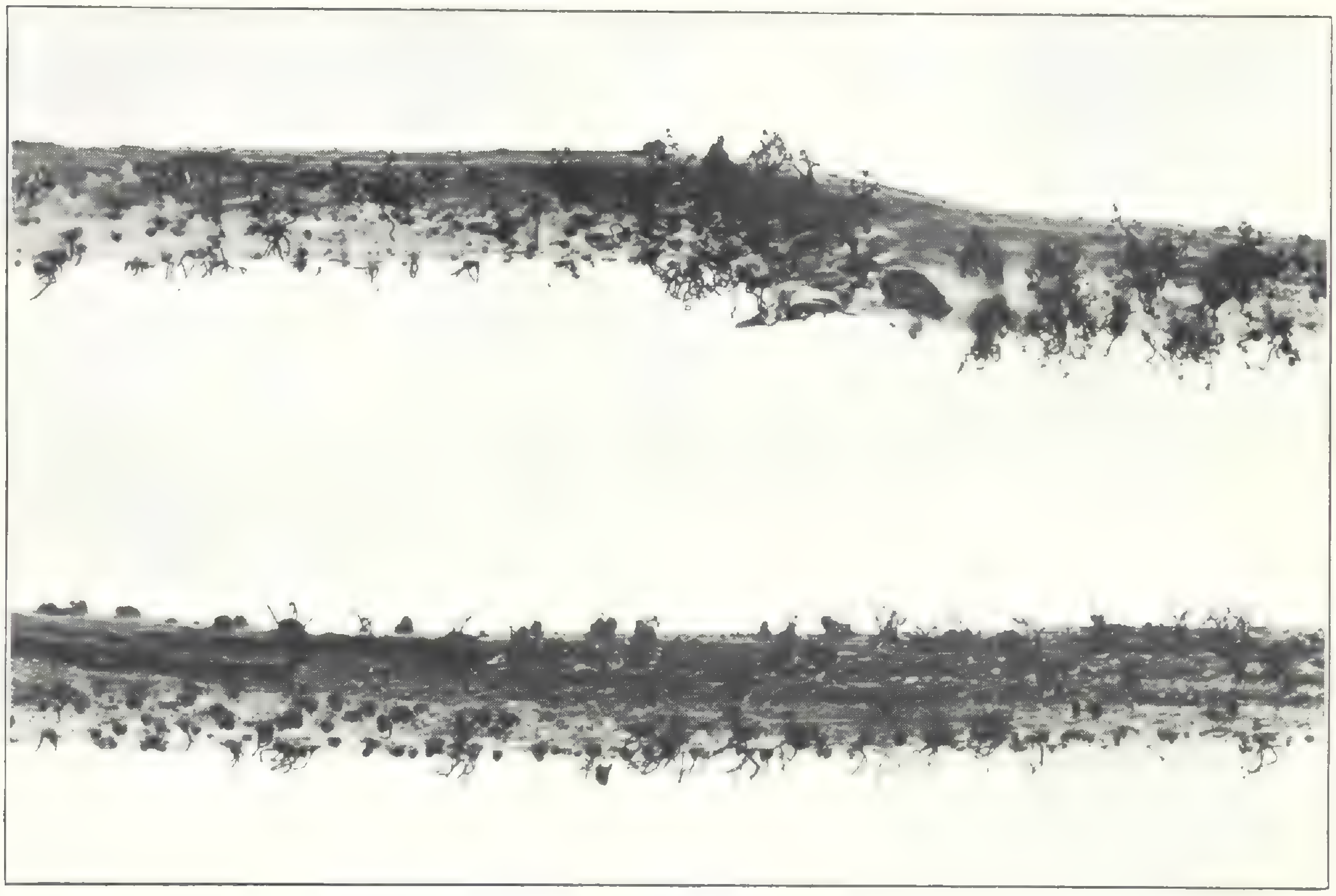


1

LEHMAN—SOYBEAN FUNGUS

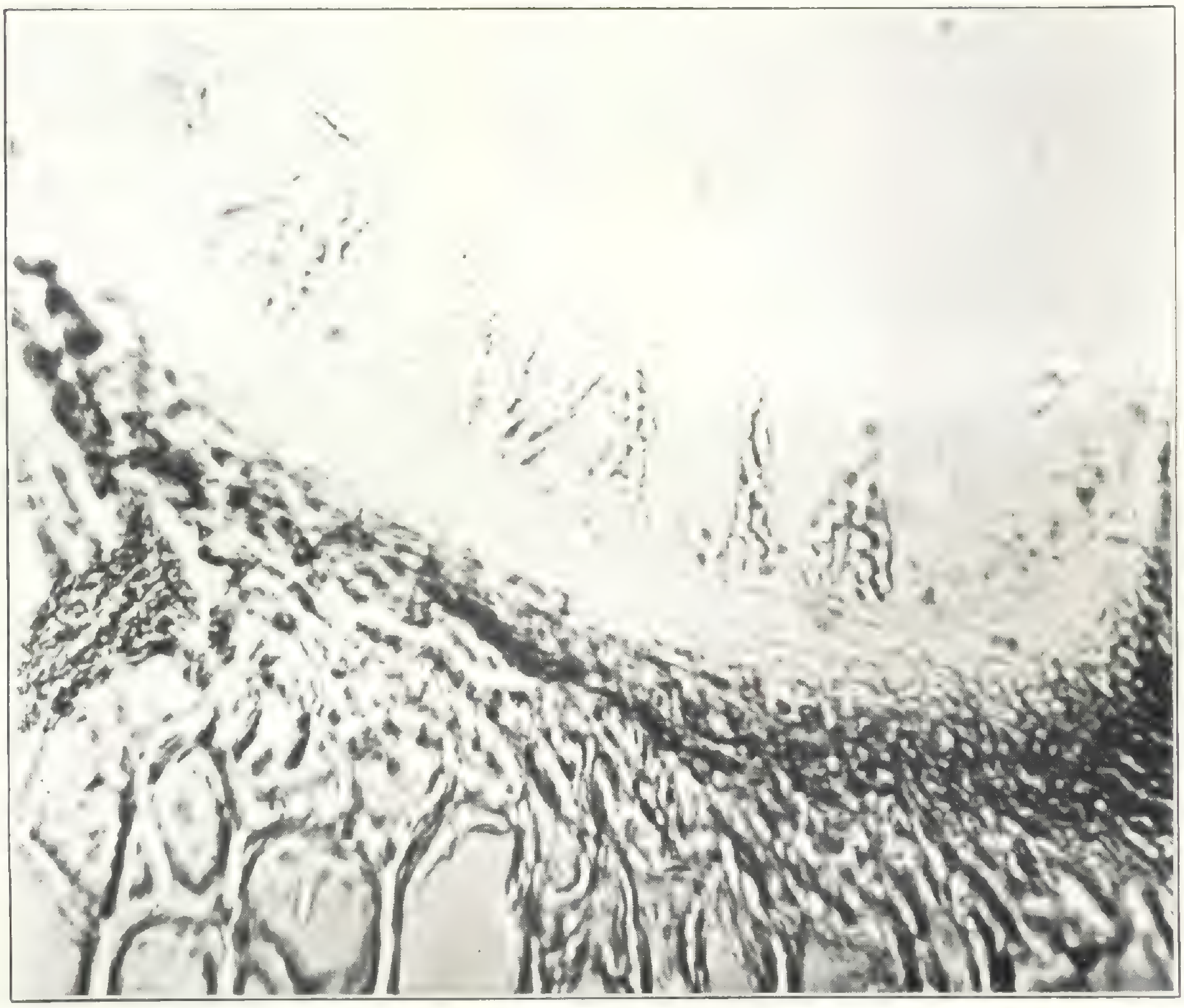
Explanation of Plate

PLATE 12

Fig. 1. At left, mycelium of *Diaporthe Sojae* growing from a disinfected embryo of a seed from a diseased plant. At right, seeds from a single diseased plant, divided into 3 lots according to their apparent degree of injury. One lot appears mouldy because of the presence of a web of mycelium over the seed-coats. Another lot is made up of seeds with badly wrinkled testas and shrunken embryos and which are for the most part incapable of germination. In the third lot the seed-coats are smooth and but little, if at all, discolored, but the greater number of the seeds are infected.

Fig. 2. Photomicrograph of a section of a perithecium of *Diaporthe Sojae* showing asci, a portion of the perithecial wall, and cells of the host tissue. Material for sectioning was grown on a petiole of a soybean leaf. Photograph by Mr. A. F. Camp.

1



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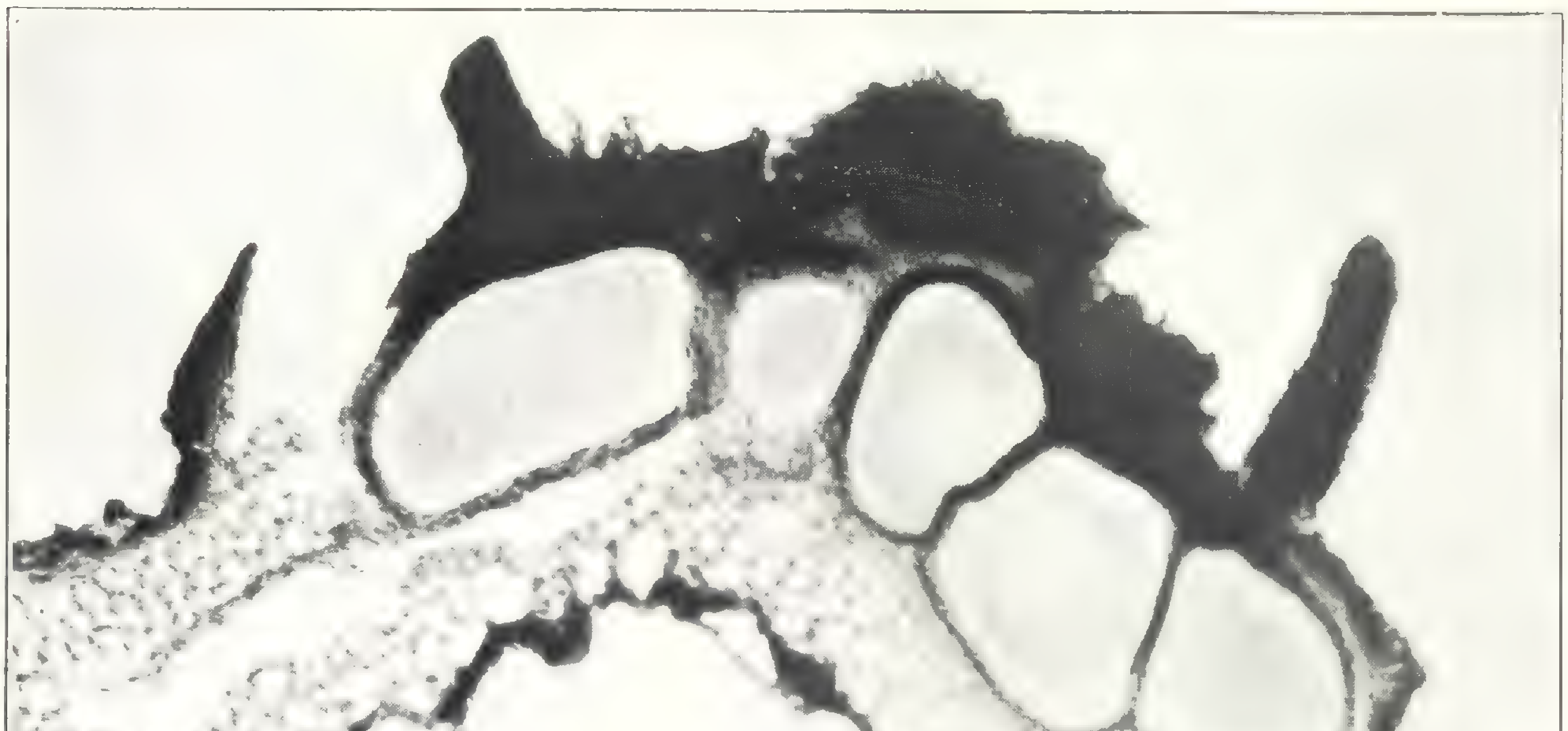
LEHMAN—SOYBEAN FUNGUS

Explanation of Plate

PLATE 13

Figs. 1 and 2. Perithecia of *Diaporthe Sojae.* Photomicrograph of a section through a stroma containing imbedded perithecia. The material for sectioning was taken from a culture growing on a sterilized petiole of soybean leaf. Photographs by Mr. A. F. Camp.

1



2

LEHMAN—SOYBEAN FUNGUS

HIGHER FUNGI OF THE HAWAIIAN ISLANDS[1]

EDWARD ANGUS BURT

Mycologist and Librarian to the Missouri Botanical Garden, Professor of Botany in the Henry Shaw School of Botany of Washington University

The fungi enumerated in the present list consist of the *Basidiomycetes* collected by Professor F. L. Stevens in the Hawaiian Islands during the summer of 1921, and of the fungi in the Bernice Pauahi Bishop Museum Herbarium which were brought to the United States for study in connection with the Stevens collections. Most of the specimens from the Museum were collected by Professor C. N. Forbes.

The 150 numbers which comprise this lot of specimens belong to 61 species; more than three-fifths of the specimens and nearly half of the species are Polypores; 11 per cent, or the 7 species, *Lepiota xylophila, Crepidotus rhizomorphus, Fomes hawaiensis, Fomes fasciculatus, Poria fasciculata, Corticium granulare,* and *Epithele hydnoides,* are regarded as indigenous. *Lepiota xylophila* Pk. and *Fomes hawaiensis* Forbes were described several years ago; the other 5 species named above are now described as new. Of the species fully determined, which occur in other countries besides the Hawaiian Islands, about 43 per cent are of cosmopolitan distribution, 13 per cent are confined to tropical regions of both America and Asia and East Indies, 24½ per cent occur in the Philippines and East Indies but not in America so far as known at present, and about 6 per cent of the species are regarded as indigenous members of the fungal flora of the north temperate region of North America. Hence the purely North American component in the higher fungal flora of the Hawaiian Islands is hardly a fourth as great as that of Asiatic, East Indian, and Philippine sources, so far as the present small number of species show.

In accordance with the instructions received, a portion of each specimen, if sufficiently ample, has been retained in the Missouri Botanical Garden Herbarium and the remainder returned to

[1] Issued October 12, 1923.

Dr. Stevens for himself and the Bernice Pauahi Bishop Museum Herbarium. The following descriptions and notes are based on characters retained by the dried specimens.

AGARICACEAE

Lepiota xylophila Peck, Torr. Bot. Club Bul. **34**: 97. 1907.

Type: in Mo. Bot. Gard. Herb., 3365, and probably in N. Y. State Mus. Herb.

"Pileus thin, campanulate or convex, umbonate, minutely squamulose, white or whitish and even on the margin when fresh, becoming brownish with age or in drying, with the umbo darker and the margin widely and distinctly plicate-striate; lamellae rather narrow, free, denticulate on the edge, minutely pulverulent, whitish, faintly tinged with yellow or greenish-yellow; stem slender, equal or nearly so, hollow, pale-yellowish or greenish-yellow; spores elliptic, uniguttulate, 8–12 μ long, 6–7 μ broad.

"Pileus 2–4 cm. broad; stem 2–4 cm. long, 2–4 mm. thick.

"On wood of red fir, Douglas fir and redwood. Hawaii. Collected by N. A. Cobb; communicated by H. von Schrenk."

Peck added further that this species is closely related to *L. cepaestipes*, from which it may be separated by its different colors, its peculiar habitat, the even margin of the fresh pileus, and its stem, which is not enlarged at the base. To this species I refer two more recent gatherings, the one without definite Hawaiian locality, *F. L. Stevens, 232;* the other, on lawn, Kamehomeha School grounds, Oahu, *Forbes & Stokes, 2196.O.* These later collections agree well with the type, but it seems probable that at least those labelled "on lawn" were growing on the ground.

Pleurotus ostreatus Jacqu.

Lanai, *G. C. Munro, 4.L;* Molokai, *C. N. Forbes, 6.M; C. N. Forbes, 13a-S.*

Pleurotus flabellatus Berk. & Br.

F. L. Stevens, 319, 668, 695, 750, 950, 997, 1149, 1151.

Pleurotus sp.

On bark, *F. L. Stevens, 592.*

A dimidiate, sessile pileus, glabrous, drying cinnamon-buff, 2 cm. long, 3 cm. broad; spores hyaline, even, 8–10 × 3½–4 μ; no cystidia.

Schizophyllum commune Fr.

On *Acacia poa*, Kauai, *C. N. Forbes, 1182.K;* Molokai, *C. N. Forbes, 6.S.a; C. N. Forbes, L 52 F; F. L. Stevens, 399,* (on Koa) *426, 549, 1118.*

Pholiota marginata (Batsch) Fr.

On ground, Maui, *C. N. Forbes, 1615.M.*

Crepidotus fulvotomentosus Peck.

F. L. Stevens, 586.

Crepidotus rhizomorphus Burt, n. sp.

Dried fructifications 5–7 mm. broad, membranaceous, sessile, pinkish buff of Ridgway, glabrous, even, the margin entire; lamellae radiating from a central point, ventricose, close, snuff-brown; spores ochraceous under the microscope, even, 6–7 × 4–4½ μ; a flattened rhizomorphic strand about 1 mm. broad, bone-brown in color, runs up the outside or within the grass culm.

On culm of an undetermined grass, Hawaiian Islands, *F. L. Stevens, 940,* type (in Stevens Herb.).

Two fructifications were present at about a centimeter apart; one was supported by a short, lateral branch of the rhizomorph, and the other had that portion of its upper surface in contact with the culm adnate to the latter, and the remaining surface free. This species is noteworthy by the thread-blight habit of its rhizomorphs.

Naucoria triscopoda Fr.

On rotten wood, *F. L. Stevens, 968.*

Agaricus ?

On dead wood, *F. L. Stevens, 1087.*

A species with pilei, 3 cm. broad, with characters too obliterated by pressure and in too fragmentary a condition for determination. Spores fuscous, even, 4½–5 × 3 μ.

Psathyra sp.

Near *Psathyra glareosa* Berk. & Br.

On ground, Kukui, *F. L. Stevens, 591.*

Stem white, hollow; spores black, even, 8–9 × 6–7 μ.

POLYPORACEAE

Polyporus arcularius (Batsch) Fr.
F. L. Stevens, 460.

Polyporus sulphureus (Bull.) Fr.
F. L. Stevens, 383, 848.

Polyporus chioneus Fr.
Mountains of Kona, Hawaii, *C. N. Forbes, 31,* in part.

Polyporus dryophilus Berk.
Waianae Range below Kolepole Pass, Oahu, *C. N. Forbes, 2034.O; J. F. G. Stokes, 8–S.*

Polyporus flabellaris Lloyd, Myc. Writ. **6**: 1035. *f. 1890.* 1921.
Maui, Aug., 1919, *C. N. Forbes, 1622.M.*

Polyporus gilvus Schw.
C. N. Forbes, 2n.2, 10–S, 11–S, 13–S; Kauai, *C. N. Forbes, 1190.K;* Molokai, *C. N. Forbes, 5.M;* on *Pithecolobium,* Oahu, *C. N. Forbes, 10–S; F. L. Stevens, 302, 410a,* (on Mango) *553, 554, 556, 557, 559, 560, 587, 1091.*

Polyporus lignosus Kl.
Molokai, *C. N. Forbes, 4.M*

Fomes Korthalsii (Lev.) Cooke as understood by Bresadola, Hedwigia **51**: 312. 1912.

An *Fomes senex* Nees & Mont.? Compare Lloyd, Myc. Writ. **4**: Syn. Fomes, 256, 259. 1917.

Slope of Mauna Loa, Hawaii, *C. N. Forbes, 14–S;* Kauai, *C. N. Forbes, 1193.K,* and an unnumbered specimen from Wapiawa Mts.; Maui, *C. N. Forbes, 17.M.*

These specimens agree with that distributed in Philippine Island Plants, *Elmer, 10646,* determined and cited by Bresadola as *F. Korthalsii.* Lloyd determined *Forbes, 17,* as *F. senex.* Lloyd does not state in his work that he has seen and compared with the type of *Fomes senex* Nees & Mont., coll. *Berteroa, 424,* in Juan Fernandez, hence it seems preferable to use for the present the name *Fomes Korthalsii. Fomes senex* has priority.

Fomes hawaiensis Forbes in Lloyd, Myc. Writ. 4: Syn. Fomes, 260, 287. 1917.

Type: *Forbes, 2.L* probably—a portion in Mo. Bot. Gard. Herb.

"Color bay, pore mouths 150 mic., otherwise as *Fomes senex.* Surely only a form, but of quite distinct form and pores one-half larger. Based on a collection from C. N. Forbes, Hawaii."

Hawaii, *C. N. Forbes* (on *Acacia*) *1075.H, 1077.H, 1079.H, 1080.H, 1081.H;* Kauai, *C. N. Forbes, 21;* Lanai, *C. N. Forbes, 2.L;* Molokai, *C. N. Forbes, 762.M, 1624.M;* Hawaiian Islands, *C. N. Forbes, 9-S, 11-S, 1076, 1629, 2116,* and *F. L. Stevens, 109, 548, 876.*

The above specimens show this species more triangular in section than *F. Korthalsii* and having a maximum thickness at the base of up to 9 cm. in specimens 5–8 cm. broad, with margin acute; pores about 4 to a mm., of the same color as the context and both between Brussels brown and antique brown; setae 30–35 $\times$ 6 μ; spores becoming colored, subglobose, even, $4\frac{1}{2}$–5 $\times$ 4 μ. The coloration of all parts and the setae and spores are the same as in *F. Korthalsii.*

Fomes fasciculatus Burt, n. sp.

Type: in Mo. Bot. Gard. Herb.

Fructification narrowly effuso-reflexed or resupinate, 8–15 mm. thick, resupinate portion up to 10 cm. in diameter, the reflexed margin 5–10 mm. broad, becoming dark quaker-drab of Ridgway, glabrous, not shining, horny, the extreme margin light buff; context and tubes drab; tubes stratified; mouths light buff to pale drab-gray, circular, 6–8 to a mm.; hyphal fascicles present in the hymenium, no setae nor cystidia; a few loose spores hyaline, even, 4 $\times$ 3 μ.

The external aspect and coloration are somewhat suggestive of *Fomes fraxinophilus.* The hyphal fascicles, to which the specific name refers, are similar to those in the hymenium of *Polystictus hirsutus* and are a constant and distinctive specific character in many other Polypores, although not heretofore recorded for this family.

On dead Koa tree, Kauai, *A. A. Heller, 2677,* type (in Mo. Bot. Gard. Herb., 4588), distributed by Heller under the herbarium name *Poria albogrisea;* Lanai, *G. C. Munro, 1.*

Fomes robustus Karst.

Hawaii, *C. N. Forbes, 28, 29.H;* Lanai, *C. N. Forbes, 1.L;* Molokai, *C. N. Forbes, 1.M;* Hawaiian Islands, *F. L. Stevens, 595,* and *J. F. G. Stokes.*

Fomes rimosus Berk.

Kauai, *C. N. Forbes, 1189.K;* Lanai, *G. C. Munro, 2;* Hawaiian Islands, Bernice Pauahi Bishop Mus. Herb., *7-S.*

Fomes Fullageri (Berk.) Cooke.

C. N. Forbes, 10; F. L. Stevens, 91, 194, 362, (on *Acacia koa*) *584.*

Fomes (Ganoderma) australis Fr.

Hawaii, on *Acacia, C. N. Forbes, 1070.H, 1078.H;* Kauai, on *Acacia koa, C. N. Forbes, 1194.K;* Oahu, *C. N. Forbes, 2115.O, 2283.O, 2415.O,* and an unnumbered specimen, *F. L. Stevens,* an unnumbered specimen on *Eucalyptus;* Hawaiian Islands, *C. N. Forbes, 8-S, 25, 2006, H. L. Lyon, 89, G. C. Munro, 3.*

Fomes (Ganoderma) applanatus (Pers.) Wallr.

F. L. Stevens, 9189; Kawailoa, Oahu, *C. N. Forbes, 2114.O.*

Fomes (Ganoderma) fasciatus (Sw.) Fr.

C. N. Forbes, 3.

Polystictus microloma Lév.

F. L. Stevens, 417.

Polystictus fibula Fr.

F. L. Stevens, 1013.

Polystictus hirsutus (Wulf.) Fr.

F. L. Stevens, 847, 850, 956, 1013; Hawaii, *C. N. Forbes, 30.H.*

Polystictus floccosus (Jungh.) Fr. ?

F. L. Stevens, 1013, in part.

Polystictus pinsitus Fr.

F. L. Stevens, 917.

Poria fasciculata Burt, n. sp.

Type: in Mo. Bot. Gard. Herb.

Fructifications resupinate, effused, adnate, thin, warm buff of Ridgway, the margin whitish and cottony; tubes unequal,

angular, 1½–2 to a mm., 1–1½ mm. long; hyphal fascicles present in the hymenium but no setae, cystidia, nor gloeocystidia; basidia simple, with 4 sterigmata; spores copious, hyaline, even, subglobose, 4–5 × 3–4 μ.

Fructifications 3–7 cm. long, 2 cm. wide. On very rotten wood, Hawaiian Islands, *F. L. Stevens, 235*, type (in Mo. Bot. Gard. Herb., 59514).

In aspect *P. fasciculata* somewhat resembles *Poria Radula* and young *Irpex deformis* but has larger pores and is noteworthy by the hyphal fascicles in the hymenium—such as are present in *Polystictus hirsutus*.

Poria sp.

Inside rotting log, Kauai, July, *C. N. Forbes, 1191.K.*

Pores circular, about 4 to a mm.; fructification too deteriorated for determination.

Trametes corrugata (Pers.) Bresadola, Hedwigia **51**: 316. 1912.

Daedalea sanguinea Kl.—*Polystictus Persoonii* Cooke.

Hawaiian Islands, *F. L. Stevens, 74, 84, 85, 86, 88, 90, 191, 380, 410, 411, 552, 580,* (on Mango) *558, 920;* Hawaii, on *Aleurites, C. N. Forbes, 595.H, 595a.H;* Kauai, *C. N. Forbes, 765.K, A. A. Heller, 2653,* distributed under the name *Polyporus cupreoroseus* (in Mo. Bot. Gard. Herb.); Molokai, *C. N. Forbes, 6-S-C, 797.M;* Oahu, on *Ficus elastica, W. T. Brigham, 6.S,* on dead *Aleurites molluccana, C. S. Judd.*

T. corrugata is probably common. When full grown it is a large bracket fungus; in section it is triangular, 9 cm. from margin to base, and with adnate base of 2–8 cm.; it is sometimes imbricated and the cluster may extend laterally 25 cm. In such specimens the greater part of the upper surface varies between vinaceous-brown and walnut-brown, with the margin and under surface and context pallid or whitish. Very young and small specimens are whitish everywhere, although very early the reddish brown color appears at the adnate base.

Trametes lactinea Berk.

Hawaii, *C. N. Forbes, 31.H;* Molokai, —, *796.Mo.*

Trametes sp.

Molokai, *C. N. Forbes, 3.Mo, 9.Mo, 10.Mo.*

Laschia cucullata (Jungh.) Bresadola, Ann. Myc. **8**: 587. 1910.

Merulius cucullatus Junghuhn, Crypt. Java, 76. 1838.—*Campanella cucullata* (Jungh.) Lloyd, Myc. Writ. **5**: Myc. Notes 58: 815. *text f. 1358.* 1919.

On wood, Hawaiian Islands, *F. L. Stevens, 963, 1036.*

HYDNACEAE

Hydnum sp.

F. L. Stevens, 1148.

Small, sessile, dimidiate pilei about 1 cm. broad. The teeth have dried resin-color, like those of *H. pulcherrimum* but probably much too small for this although very immature. No hymenium yet present.

Odontia Wrightii (B. & C.) Pat.

F. L. Stevens, 966.

Easily recognized by the egg-yellow color of the fructifications. Common in North America and occurs also in South America and Japan.

Odontia?

On Kukui, *F. L. Stevens, L 397.*

The teeth do not retain their tips in my sections: hence this may be a resupinate *Hydnum.*

THELEPHORACEAE

Cyphella villosa (Pers.) Karst.

On dead stems of *Pipturus, F. L. Stevens, 589.*

Hymenochaete tenuissima Berk.

On decaying wood, *F. L. Stevens, 118, 967.*

This is the only pileate species of *Hymenochaete* received; the thin and pliant, sessile, tobacco-colored pilei make this easily recognizable.

Hymenochaete spreta Peck.

On frondose wood, *F. L. Stevens, 877.*

Hymenochaete cinnamomea (Pers.) Bres.

F. L. Stevens, 871, 878.

Always resupinate, like *H. spreta,* from which its most obvious difference is in not cracking in drying.

Stereum elegans (Mey.) Lloyd? See Burt, Mo. Bot. Gard. Ann. **7**: 105. *pl. 3, f. 15.* 1920.

On rotten, moss-covered wood, Oahu, Oct., 1911, *C. M. Cooke, 9-S.*

I should refer these specimens more confidently to *S. elegans*, had they been found growing on the ground and with the stem not glabrous.

Stereum latum Cooke & Mass. Grevillea **20**: 92. 1892; Sacc. Syll. Fung. **11**: 121. 1895.

F. L. Stevens, 236.

S. latum was described from specimens collected at Perak, Malay Peninsula, with my preparations and notes of which the Hawaiian specimens agree closely. The specimens have some resemblance to *Stereum cinerascens*, but are thinner and form large, easily separable, effuso-reflexed sheets, with the reflexed portion standing out 2–3 cm. and extending laterally more than 10 cm.; upper surface is strigose-hairy and concentrically sulcate; hymenium somewhat avellaneous when received and with a distinct tinge of pink suggestive of *Eichleriella Leveilliana;* cystidia incrusted, 30–50 × 11–16 μ; spores hyaline, even, 11 × 6 μ; KHO solution bleaches the sections and becomes somewhat vinaceous in their proximity by some substance which it dissolves.

Aleurodiscus perideniae (Berk. & Br.) Henn.

On dead wood having a large pith, E. Maui, July, 1910, comm. by F. L. Stevens, 26.

The fructifications are very beautiful, having the aspect of those of *Stereum ochraceo-flavum* but a more orange hymenium and wholly different structure.

Corticium arachnoideum Berk.

On dead stems of *Cibotium, F. L. Stevens, 964.*

Corticium granulare Burt, n. sp.

Type: in Mo. Bot. Gard. Herb.

Fructification effused, adnate, snow-white, pulverulent under a lens, very thin, only 15–30 μ thick, not bearing a continuous hymenium but consisting of bushy branched, suberect hyphal

clusters standing out from the substratum and near together, with their main trunks up to 6 μ in diameter and short-celled; no cystidia nor gloeocystidia; basidia simple, 15 × 4½ μ, with 4 sterigmata; spores hyaline, even, flattened on one side, 4–4½ × 3–4 μ, copious.

Fructifications scattered along the substratum, 1–3 cm. long, 4–8 mm. wide.

On dead herbaceous stems, Hawaiian Islands, *F. L. Stevens, 381*, type.

Epithele hydnoides Burt, n. sp.

Type: in Mo. Bot. Gard. Herb.

Fructifications effused, adnate, drying pallid to olive-buff of Ridgway, very thin, 30 μ thick, with often not more than a basal cell between each basidium and the substratum; hymenium a plane surface parallel with the substratum and bristling with conical hyphal fascicles about 10 to a mm. which start from the substratum; hyphal fascicles 90 μ long, 20–30 μ in diameter at the base, with crystalline matter in the axis; basidia simple, 20–30 × 12 μ, with 4 large, divergent sterigmata; spores hyaline, globose, 9 μ in diameter, copious along the surface of the hymenium but none seen attached to the sterigmata.

Fructifications in numerous small patches 5 mm.–2 cm. long, 2–4 mm. wide, often becoming confluent.

On dead stems of *Cibotium*, Hawaiian Islands, *F. L. Stevens, 957*, type.

Under a lens the fructifications have the aspect of a whitish, resupinate *Hydnum* but the hymenium does not clothe the organs resembling teeth. The whitish color, large, globose spores, and large basidia distinguish *E. hydnoides* from other species of the same genus.

AURICULARIACEAE

Auricularia auricula-Judae (L.) Schrt.

F. L. Stevens, 379, 412, 413.

Auricularia nigrescens (Swartz) Farl.

Hirneola nigra Fr.—*Exidia polytricha* Mont.

F. L. Stevens, 89, 590; H. L. Lyon, in Sydow, Fungi Exot., 322, under the name *Auricularia nobilis;* Kauai, *C. N. Forbes,*

1184.K; Molokai, *C. N. Forbes, 5.M, 6.M;* cited from Oahu by Fries, Nov. Symb. Myc. 118. 1851

Auricularia tenuis (Lev.) Farl.

F. L. Stevens, 194a, 919, 943; cited from Oahu by Fries, Nov. Symb. Myc. 118. 1851.

This species differs from the preceding one in becoming glabrous; Nos. 919 and 943 show this change in progress, in 194a it is completed.

GASTEROMYCETES

Mycenastrum Corium (Guers.) Desv.

Slopes of Mauna Loa, Hawaii, *C. N. Forbes, 16-S.*

Myriostoma coliforme (Dicks.) Cda.

Geaster coliformis (Dicks.) Pers.

South of Omaopoili and near flow of 1843, 6300 ft. altitude, Hawaii, *C. N. Forbes, 1072.H.*

Lycoperdon cepaeforme (Bull.) Lloyd.

On lawn of Kamehameha School grounds, Oahu, May and September respectively, *C. N. Forbes, 2195.O, 2269.O.*

Lycoperdon gemmatum Batsch.

Maui, *C. N. Forbes, 1616.M.*

Lycoperdon Wrightii B. & C.

Aina Hoa, altitude 6000 ft., Hawaii, June 21, *C. N. Forbes, 906.H.*

Lycoperdon sp.

On *Sadleria* trunks, ridge, Mokai Gap, Kauai, *C. N. Forbes.*

PYRENOMYCETES

Ustulina vulgaris Tul.

Conidial stroma differentiating perithecia. This stage is often mistaken for a *Stereum.*

F. L. Stevens, 587.

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INDICATIONS RESPECTING THE NATURE OF THE INFECTIVE PARTICLES IN THE MOSAIC DISEASE OF TOBACCO[1]

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Among plant pathologists there is to-day no topic of more engaging interest and no problem more difficult than that of the nature of the causal agency in mosaic and allied plant diseases. Possibly we might extend this statement so as to comprehend at the same time the causal factors involved in those types of vegetative variegation, or modified pigmentation, whether infectious or not, which afford the decorative mottled, spotted, and striped plants so much cultivated for foliage effects.

At various times almost every conceivable view has been held as to the nature of the etiological agent in tobacco mosaic, but from the earliest experiments it has been perfectly clear that the disease is transmissible. The great majority of workers have accepted the evidence of the filterable character of the infective agency, and it is this which gives to the disease much of its peculiar interest.

The relation of the true mosaic diseases to certain other types of

[1] This paper was read at the annual meeting of the American Philosophical Society, Philadelphia, April 21, 1923.

plant disease involving chlorosis, whether with or without mottling, has not been positively determined, but, for the most part, there are some common characteristics of all to which at least passing reference must be made in the course of this paper. The relation of mosaic diseases to infectious or non-infectious "natural" variegation (yellow and green) of foliage plants also remains for detailed study. In this paper we propose to discuss more particularly some of the problems relating to infectious chlorosis with special emphasis on that type illustrated by the mosaic disease of tobacco.

The true mosaic "diseases" of dicotyledons constitute a somewhat homogeneous group, for they exhibit a blotching or mottling —defined as a mosaic—in which usually both a hypoplastic and a hyperplastic development of the tissues ensues. The mottling is very largely confined to the leaves and may be characterized by regions of lesser chlorophyll development (often a definite yellowing or chlorosis) and regions of intensified chlorophyll development. The latter has sometimes been treated as a quantitative intensification of the chlorophyll and the former as a degradation or diminution of chlorophyll. In discussing the symptomatology of such diseases it has been more the custom to emphasize as the disease the chlorotic areas; and certainly if the sugar cane, maize, and certain other monocotyledonous "mosaics" are included in the category of true mosaics, then the chlorotic areas are admittedly strongly diseased. Abnormal greenness may be, nevertheless, so characteristic that this too should have its place among the symptoms.

Among those who have studied tobacco, bean, and similar mosaics the view has been held also that the intensified green areas are primarily those of disease. In the studies of Dickson ('22) stress is properly laid, it seems to us, upon both aspects of chlorophyll change. Here, as in the earlier important work of Iwanowski ('03), it is clearly shown that in the leaves intensified greenness is correlated with, and in part (at least) due to, increased development of chlorophyllous tissues,—hyperplastic changes. This condition prevails as a part of the differentiation which is produced in young leaves, or in leaves formed after infection. There is no such result in organs already mature at

the time of infection. Woods ('00, '02), Chapman ('17), and others have also given attention to the anatomy of mottled areas. The yellow or chlorotic areas are ordinarily correlated with regions of lesser development of the chlorophyllous tissues,—with hypoplastic development. It is of some interest to note that in a typical mosaic disease of swiss chard observed by the senior writer at the Missouri Botanical Garden in 1919 the chief, if not sole, visible color effect was intensified greening in a blotched pattern. In general, there is in dicotyledonous mosaics a focal distribution of effects, and possibly it may be strictly analogous to conditions in human measles, or to the effects produced by an injection into the body of diphtheria antitoxin, or to the focal distribution of pigment in certain skin diseases. In the mosaic disease of tobacco, necrosis, as generally understood in plant pathology, does not occur.

It should not, however, be assumed that marked mottling is necessarily a symptom of these diseases, since many plants or plant species, not themselves seriously mottled, may exhibit, through infection experiments, evidence of severe attacks of the disease so far as this may be expressed through the infectiousness of their juices. Moreover, dwarfing of the general plant, spindling shoots, abscission of blossoms, and many other characteristics are typical of mosaic diseases as they are understood in certain plants, notably in the potato and in certain cucurbits.

Mosaic diseases are recognized to occur in many species of *Solanaceae* (night-shade family), *Cucurbitaceae* (gourds, squashes, etc.), *Leguminoseae* (peas, clovers, etc.), *Chenopodiaceae* (beet and spinach family), *Rosaceae* (raspberries, etc.), *Gramineae* (grasses, sugar-cane, etc.), and many others, altogether about 20 families.

There have been several possible views as to the nature of the causal agency or agencies in mosaic diseases, all or nearly all of which have been exploited, and perhaps very nearly discarded.

ENZYME THEORY

The chief adherent of the enzymic nature of mosaic disease has been Woods ('99, '00, '02). He postulated that the cause of mosaic may be found in an enzyme disturbance in which the

chlorotic condition might result from an extensive development of oxidizing enzymes. Unfortunately, this would not explain the condition prevailing where the tissues are hyperplastic, though, if found consistent, it might explain the hypoplastic relation. Moreover, the suggestion that oxidase inhibition upon diastase would explain the accumulation of starch in diseased tissues does not well apply, since the starch accumulation has been shown by Freiberg ('17) and Dickson ('22) to be in the greener areas, a finding confirmed by ourselves. Finally, Allard ('16) has convincingly demonstrated that the infective agency and oxidase are not the same, for a differential and quantitative destruction of the oxidase does not affect infectivity, whereas it is possible also to destroy the active agency in the mosaic disease and yet demonstrate oxidase action. Woods' viewpoint has been adopted also by Heintzel ('00), and in part by Chapman ('13). After criticising (Hunger, '03) the oxidase theory of Woods, Hunger ('05) regards the disease as a nutritional one possessing the peculiar property of being "physiologically autocatalytic," acting by contact and also able to regenerate itself. In some work done in this laboratory Freiberg ('17) also advocated the enzyme viewpoint rather than the more general virus effect, but he considered the enzyme to possess none of the nature of oxidases. The idea that it may be an enzyme was based in part on its absorption by talc, on the specificity of the reaction between the mosaic agency and formaldehyde, and likewise on the basis of the resistance of the body to antiseptics in general. Just how the reproduction of such an enzyme might be accomplished was not considered in detail, but was accounted for on general physiological grounds. In this connection attention was drawn to the fact that upon the injection of the toxin of *Bacillus diphtheriae* into a healthy patient, the usual pathological condition results, that is, the production of lesions characteristic of that disease, apparently with the production of additional toxin in the system.

THE BACTERIAL THEORY

In one of the earliest of the scientific reports on the mosaic disease of tobacco, that of Mayer ('86), bacteria were regarded as the causal agency, although no satisfactory proof was afforded.

Iwanowski ('03) describes the presence of bacteria-like as well as amoeba-like bodies within the tissues. The bacteria were described as intracellular, occurring in the vicinity of the cell wall, and extremely minute in size. Being the first to demonstrate the filterable character of the tobacco mosaic agency, it is rather interesting that he (Iwanowski, '92) ultimately concluded that bacteria were causally related to the disease. Strangely enough, he did not employ the skill in determining this point that he applied to other aspects of the problem. In recent years, in spite of the rapid advances in the culturing of bacteria, the bacterial view has gained few, if any, consistent adherents. A small nitrate-reducing streptococcus found in mosaic-affected tobacco is briefly referred to by Boncquet ('16, '17). Bacteria-like bodies were also identified by Dickson ('22), and he endeavored to culture the organism. By his method, bits of affected leaves were cut out, and after short disinfection intervals in alcohol and mercuric bichloride, crushed in tubes of bouillon. As clearly recognized by him there could be no certainty that surface organisms were killed. Dickson, however, secured infection by inoculation from these tubes after clouding occurred. Similar results, as he indicates, might be obtained by this method, in view of the amount of the agency originally inserted, whatever the nature of the infective particles, and the clouding with bacteria may have been entirely from secondary or surface forms.

It is rather significant that there are so few adherents of the bacterial nature of mosaic diseases. On the other hand, there is no great amount of published evidence against the bacterial viewpoint. This is certainly not wholly due to lack of effort to find bacteria, but rather to two facts: first, in at least half a dozen cases personally known to the writers, where extensive studies were made, the negative evidence was considered of too little consequence for publication; and second, acceptance of the filterable organism view tended to discourage search for bacteria. The bacterial view may be regarded at present as wholly unsustained.

THE VIRUS OR FILTERABLE VIRUS THEORY

The connotation of "virus" is fairly definite, inasmuch as the term is now generally restricted to homologize with a filterable

agency of disease. In the present paper we shall use the term virus in the general sense just referred to. The ability of the agency or "organism" to pass through the pores of a standard Berkefeld or Chamberland filter is the usual criterion. Some would undoubtedly define a virus as an ultramicroscopic organism, probably of bacterial nature. This, however, was not the final view of Beijerinck ('99a) who postulated a "contagium vivum fluidum" as the cause of the mosaic disease of tobacco. While his agar filtration studies may now be regarded as inadequate, the capacity of the infectious agencies of several mosaic diseases to pass through certain standard filters under certain conditions has now been demonstrated by Iwanowski ('92), Beijerinck ('99), Allard ('16), Doolittle ('20), Duggar and Karrer ('21), and others. After summarizing an interesting study of the properties of the virus of tobacco mosaic Allard ('16a) is convinced that "there is every reason to believe that it is an ultramicroscopic parasite of some kind." If this evidence of the "filterable" nature of the disease is admitted, it would bring the causal agency into a class possibly composed of a rather miscellaneous group of bodies, since there are well-known analogies in the agents of animal disease. The "virus" view in one form or another has been widely held, but the favorite idea has been an ultramicroscopic organism.

THE AMOEBA OR PROTOZOAN THEORY

It has been pointed out that both Iwanowski and Hunger drew attention to the presence in mosaic plants of bodies which they interpreted as amoeba-like. These, however, were of relatively infrequent occurrence. More recently, in a study of the mosaic disease of sugar cane in the tropics, Matz ('19) has found certain cells of the affected tissue filled with a granular matter. Using reliable cytological methods, Kunkel ('21) confirms the presence of such cells in a mosaic disease of corn, to which, however, he seems very justly to attach no significance. Kunkel does find in the cells of diseased corn peculiar plasma-like bodies in the vicinity of the nucleus. He has also been able to distinguish similar structures in the "diseased" areas of *Hippeastrum* (Kunkel, '22) also affected with a mosaic disease. This is a very clean-cut

demonstration of a plasma-like body, but whether or not it may be a modified cell structure, a pathological by-product, a colony of granular bodies, or something else, is not yet clear, nor is its significance in relation to the "mosaic" disease of these monocotyledons known.

A study of tobacco mosaic in Sumatra by Palm ('22) brings casual reference to some abnormal structures. He refers very briefly to cytological work on this mosaic and mentions the occurrence of corpuscular bodies more opaque than the general protoplasm. Likewise, he notes the occurrence of "a second foreign cell element, consisting of extraordinary, small granules." With this hazy evidence he proceeds to relate the bodies to the "so-called corpuscles of Gardner" and concludes that a "Strongyloplasma" species must be considered as the cause of the disease. Indeed, he designates the "organism" *Strongyloplasma Iwanowskii*, promising a more extended publication.

The sensation of the joint meeting of the Botanical Society of America and the American Phytopathological Society at Boston in December, 1922, was a report by Nelson on "The Occurrence of Protozoa in Plants Affected with Mosaic and Related Diseases." The stage was well set for such an announcement. The titles of several papers arranged for that same meeting indicated the finding of unusual structures in the cells of several plants affected with mosaic-like diseases. The careful work of Kunkel ('21, '22) referred to earlier in this paper; the observations of Matz ('19), Palm ('22), and Dickson ('22); the attention recently bestowed upon the existence in the spurge and milkweed families (Lafont, '10; França, '20; and Mesnil, '21) and other dicotyledons (Franchini, '22, '22 a-g) of flagellates normal to the latex tubes;—these considerations all served to establish an atmosphere on that stage exceedingly favorable or impressionable in respect to protozoology. Under such conditions Nelson described or presented upon the screen in the form of photomicrographs evidence for the existence in bean mosaic of 6 principal forms or types of a protozoan organism alleged to occur in the phloem of a diseased plant. It is impracticable here to take the time to indicate the characteristics of most of these types of flagellates described. The paper has since appeared as a technical bulletin of the

Michigan Agricultural Experiment Station (Nelson, '22). Besides the bean mosaic, similar diseases of clover and tomato, and the leaf roll of potato are reported replete with protozoans. It may not have appeared remarkable at the time the paper was presented but it is significant now that Nelson gave no picture of the conditions in the comparable cells of healthy plants. In two places in the printed paper (Nelson, '22) he refers to healthy tissue, one reference being to the potato, where he says, in part, "No organisms have been found in the sieve tubes of these plants in all the slides examined;" whereas in a general discussion of relationship he affirms that "the finding of definite protozoan organisms in constant association with mosaic plants and their absence from healthy ones indicate that they are probably the factor so long sought as the cause of these diseases."

We have endeavored to supplement this work with an elaborate cytological study of healthy and diseased tobacco and tomato tissue, healthy bean tissue, and healthy cucurbit tissue. In the Solanaceous plants we find in the phloem and in other elongated cells of perfectly healthy individuals precisely the same bodies that are found in diseased tobacco and tomato plants. The number of cells with such inclusions is not great. The most characteristic of these bodies are often sinuous, or screw-like, also of other types. They are usually homogeneous and often appear to be waxy in nature. Some supernumerary nuclei or cytoplasmic aggregates are also observed, and the remains of plastids may be associated with these. We have not studied diseased bean tissue, but in healthy tissue the long cylindrical, ovoidal, or elliptical bodies are generally homogeneous in character, centrally disposed, and frequently associated with cytoplasmic strands, the latter giving the appearance of one or more flagellae at the ends. It seems apparent that these particular bodies are those that have been described by Strasburger in normal sieve tissue. In certain cucurbits, notably in *Chayote edule*, disintegrating plastids in cells undergoing rapid elongation present the appearance of organisms of various types, all more or less nodose. It seems unnecessary to describe these bodies further in the present connection. It is clear that the peculiar structures portrayed by Nelson are all to be found, but our claim

is that essentially all of these may be paralleled in perfectly healthy tissue. Moreover, the relations of these bodies seem in no way to suggest flagellates that may be normal to the tissues, whether diseased or healthy. From our studies we are convinced that these "flagellates" are made up of several factors, and while we have not attempted a careful micro-chemical examination, nor made a complete study of the developing tissues, such possibilities as the following may be noted: elongated masses of gummy material long known to be characteristic of certain sieve tissues; cytoplasmic aggregations or areas of contraction possibly associated with disintegrating plastids; elongate, accessory, and perhaps disintegrating nuclei; and homogeneous aggregates of unknown origin, possibly of waxy nature.

The importance of the problem justifies the feeling that a complete reinvestigation of these cell phenomena is being pursued by many others, and that out of it may come some compensating observations that will throw light rather than shadow on the nature of the mosaic diseases.[1]

ULTRAFILTRATION EXPERIMENTS

The fact so frequently confirmed that the agency of mosaic disease passes freely through the pores of the average Berkefeld or Chamberland filter did not establish, prior to 1921, the size of the infective agency further than to indicate that it is considerably less than that of the usual plant or animal pathogen. It sufficed merely to relate the agency to filterable organisms. In order ultimately to determine more accurately the relations of this agency it seemed essential to make a detailed study of its size relations. This was done by the present writers (Duggar and Karrer, '21), and reference to this work is a necessary preliminary to the further results which will be reported and to the theoretical considerations which we wish to present.

The work referred to consisted first in securing graded series of ultrafilters, some of which should permit the infective particles to pass freely (as shown by the infectiousness of the juice) and

[1] Since the oral presentation of this paper, much additional light has been thrown upon the distribution and relations of these abnormal bodies described by Nelson, and similar structures, in a series of papers published in Phytopathology, Vol. 13, No. 7, 1923, by the following authors: (1) Kotila and Coons; (2) Doolittle and McKinney; (3) Kofoid, Severin, and Swezy; and (4) Bailey.

others with pores or lacunae so fine as to prevent or greatly inhibit the passage of such particles. A second phase of the work involved a careful technique in the use of the ultrafilters. A third phase required the inoculation of healthy plants with the various filtrates obtained in order to determine the percentage of dilution of the particles, if possible. Finally, some method of standardization of the filters was necessary whereby their capacity to permit or prevent the passage of particles might be related to colloidal particles of known, or approximately known, sizes. It will be unnecessary to go into the details of these experiments. Two aspects of the results require emphasis. It was possible to find a filter, in this case a cylindrical, porcelain atmometer cup, which in a given interval of time, at a given pressure, and at the reaction of the diseased tobacco juice, permitted only a relatively small number of the infectious particles to pass through. Considerable dilution of the juice from the standpoint of these particles was therefore effected. This was shown by a reduction in the incidence of infection from 90–100 per cent in the usual controls to 5–20 per cent in the case of the porcelain filter only partially permeable to the infective particles.

Standardization of the filters was accomplished by the use of hydrophilic colloids of biological origin. These were selected in preference to sols of inorganic origin, such as gold sols, because of possible greater complications (when employing the latter) arising from electrical relations. The series of organic compounds employed included casein, gelatin suspensions, lactalbumin, hemoglobin, and dextrin. Fortunately, this series sufficed. The results indicated that the hemoglobin content of a standard hemoglobin solution prepared from fresh ox blood was diluted to a very considerable degree in passing through the same filter which obstructed to a large degree the passage of the infective particles. In experimenting further with substances on either side of hemoglobin, in reference to size range, it was clear that from such filtration experiments the deduction must be made that the infective particles of mosaic disease approximate in size those of a fresh 1 per cent hemoglobin solution.

The best data on the size relations of hemoglobin particles indicated a diameter of approximately 30 $\mu\mu$. It is presumable

that we are dealing in the case of a colloidal solution with particles and not with molecules. This particle size is to be compared with an average short diameter of about 1000 $\mu\mu$ for many pathogens.

If we are dealing with an organism, that is, an organized ultra-microscopic individual of approximately 30 $\mu\mu$ in diameter, its life relations must be very different from those of an organism whose volume relations are to this as 37000 to 1 or about 1,000,000 to 26. This would be the relation between the average bacterial plant pathogen and the mosaic virus. Assuming a complex organization, many theoretical questions would arise for consideration. Among these might be mentioned perhaps above all that of the surface tension conditions in such a structure, also the possibility of organization at all (membrane existence, etc.) as now comprehended.

The filtration work has been repeated with scrupulous care and it has led to results similar to those above described,—invariably pointing to an infectious particle with a size approximating that of fresh 1 per cent hemoglobin. A question which then forces itself upon the attention is: What is the peculiar nature of such a particle? To arrive at a tentative answer to this question, it would be necessary to consider all known properties of the agency, to analyze the data already carefully worked out, to plan many experiments of an entirely new type with a view to determining the behavior of the body concerned, and to contrast the inception and course of the mosaic disease or related phenomena of chlorosis in other plants. As far as possible it would be essential to examine also any possible relations of the viruses of animal diseases that may assist in one or more general interpretations.

Under the most favorable growing conditions the period of incubation of the tobacco mosaic is from 10 days to 2 weeks. By period of incubation, in this connection, is meant the time required for the development in the young leaves of the infected plant of unmistakable symptoms of mottling. In this interval of time the infective agency is widely distributed in the plant. It is not confined to the leaves (young) capable of exhibiting pronounced or favorable mosaicing, but may be found in older leaves, roots, etc. It has in reality a phenomenal power of "migration" from cell to cell,—a power none the less pronounced

even if the vascular system should be shown to constitute one of the paths of this migration. Moreover, the power of migration is not a matter which may be easily determined.

The rapid and almost complete distribution of the organism in the tissues accords well perhaps with a body minute in size and attenuate in form, or else a body so fluid in character as to be capable of assuming an extremely attenuate form. A living structure so pliable and attenuate might be expected to be sensitive to reagents and conditions. To a considerable degree this sensitiveness is not true of the virus of mosaic. In our experiments it resisted the usual procedure of dehydration by means of acetone and alcohol. Modifications of the Buchner method for the extraction of enzymes (zymase) from yeast cells were applied to a pulp of fresh, diseased, leaf tissue ground with very fine quartz sand, 5 parts of the former to 1 of the latter. Three series of experiments were arranged. In the first, the pulp was treated with full-strength acetone. There were 2 treatments, each of 3 minutes, the tissue being drained after the first addition of acetone and then fresh acetone applied for an equal interval. Finally, the material was dried as promptly as possible under an electric fan. In the second case the dehydration treatment consisted in the addition of 95 per cent alcohol for a 3-minute interval, followed by pure acetone, and finally dried, as above. In the third case the treatment was 95 per cent alcohol, followed, in the same intervals as before, with 98 per cent alcohol, and finally dried. After 3 days these residues were extracted with water, each for 1 hour, using about 10 parts of water to 1 of the dried material. After the filtration of each extract through cotton, 20 plants were inoculated with each extract, and suitable (20) controls were maintained. These plants were under favorable growth conditions, and they exhibited the symptoms of disease promptly. At the end of 19 days, 1 healthy plant only remained in each set. All uninoculated controls remained healthy, and the incidence of disease in the control which was inoculated with diseased juice was likewise 19.

When, however, the amount of alcohol and acetone was greatly increased in relation to the bulk of material used (approximately 200 times as much), the incidence of infection was low, showing

that the infective particles do not withstand complete dehydration.

A study of the effects of longer exposure to various grades of alcohol has been carried out at some length by Allard ('16a), who found that relatively speaking the infective properties are quickly destroyed by the higher strengths of ethyl alcohol. He indicates destruction by 80 per cent alcohol in 30 minutes. On the other hand, in later work, he (Allard, '18) has shown some striking resistance of the infective agency to the weaker grades of alcohol. We may note a few instances. Kept in 25 per cent alcohol 34 days and then inoculated into the host, 7 out of 10 plants developed mosaic; this, however, is obviously exceptional, since in another test the virus kept in 25 per cent alcohol 199 days yielded 7 out of 10 diseased plants; in 50 per cent alcohol after 40 days no disease was induced; in 50 per cent alcohol after 35 days 4 out of 10 plants became diseased. With another sample of the virus in 50 per cent alcohol for about 5 days only 2 plants were diseased after inoculation from this material.

From a comparison with active cells in the vegetative condition it will be seen that these results indicate a high degree of resistance since the average bacterial cell may be injured after 24 hours by a concentration of from 5 to 10 per cent alcohol. Moreover, the yeast cell, which shows a specific tolerance of alcohol concentration, is itself injuriously affected by more than about 15 per cent alcohol. If the comparison is made with the tolerance of the spores of certain species of bacteria, we shall find that the infective particles of mosaic are less resistant. Feeling that it was unwise to accept some of the data which have been published on this point, we made a study of the tolerance of the spores and vegetative cells of the hay bacillus, *Bacillus subtilis*.

In the duplication of this work the senior author was assisted by Dr. H. R. Rosen. One cubic centimeter of a dense infusion of this organism was placed in a series of alcohols diluted with a decoction of tobacco juice so as to get respectively 10, 20, 30, 40, and 50 per cent alcohol, and similar concentrations of acetone. At intervals up to 10 days, streak cultures from these concentrations of the disinfectant yielded in each case continuous growth of the organism. Similar cultures were made with a young

culture of the bacillus in which, of course, there were relatively few cells in the spore condition. In the latter case very few colonies appeared after the third day. In a repetition and amplification of this work, the tobacco extract was not employed, and the organism was suspended in concentrations of alcohol, as follows: 10, 20, 40, 60, and 100 per cent, with controls in bouillon and in distilled water. From all those cases in which the spore suspension was employed, a profuse growth was obtained on every streak culture from 10 to 99 per cent alcohol, with apparently no lessening of the intensity of growth as between 10 per cent and 99 per cent. In the case of the acetone-treated material, profuse growth was attained at 10, 30, and 60 per cent acetone with some indication of less intense growth in absolute acetone.

Following the above observations, made the third day, it was determined to make isolation cultures after the tenth day, and these were accordingly arranged and a careful count made as the colonies appeared. There was a progressive diminution in the number of cells alive from the 20 per cent to the 99 per cent alcohol. For that whole interval, however, this decrease amounted to only about nine-tenths of the organisms present in the infusion. There was scarcely any diminution as between 10 per cent acetone and 60 per cent, but in absolute acetone the number of organisms was considerably reduced. These data confirm the statement previously made to the effect that the virus of mosaic is less resistant than certain spore forms of the bacteria. This, however, is not surprising, for whatever may be the nature of the virus, many colloids lose to a considerable degree their hydrophilic character when treated with strong alcohol. The point of interest, therefore, is more particularly the nature of the bacterial spore which permits survival in the high concentrations discussed, a problem rather apart from our specific investigation.

EFFECT OF GRINDING ON THE INFECTIVITY OF THE TOBACCO VIRUS

Inasmuch as the thermal tolerance and the resistance toward dehydrating and disinfecting agents, while suggestive, did not seem to set off this virus as possessing properties peculiarly distinctive, it seemed particularly desirable, in view of the size

relations, to determine the influence of long-continued grinding under conditions which are generally effective in disrupting living cells. A final series of experiments in this field will be sufficient to indicate the relations encountered. It should be indicated, however, that this series is in accurate accord with less extensive work previously undertaken to determine the same point. The grinding was carried out in an agate mortar with motor-driven, excentrically arranged pestle, the usual device employed in grinding bacterial cultures. Equal amounts by weight of fresh leaf material and diatomaceous earth were used.

INOCULATION EXPERIMENTS WITH FINELY GROUND MATERIAL FROM DISEASED TOBACCO LEAVES. INTERVAL, 3 WEEKS

Nature of inoculum	Total diseased after 4 weeks (ten plants inoculated)
Ground 3 hours	8 plants diseased
Ground 9 hours	6 plants diseased
Control, no inoculation	None diseased
Control, fresh dis'd. juice	7 diseased

While there has been some inconsistency in the data from other grinding experiments they point in general to one conclusion, namely, that the virus is highly resistant to protracted grinding with diatomaceous earth when the virus is ground with fresh leaf pulp. It is less resistant when filtered through porous cups, then mixed with diatomaceous earth, and ground for 9 hours. The presence of leaf material acts to prevent the greater injury. In order that these experiments may be significant it is necessary to compare the grinding of the tobacco virus with that of a species of bacteria.

For this purpose also we have employed *Bacillus subtilis* in the spore condition. Two cc. of a 22-day-old culture in bouillon (rich in spores) were thoroughly mixed with 2 gms. sterile diatomaceous earth in a petri dish. This mixture was then dried, being protected during drying by sterile paper bags. It was then subjected to grinding for the same intervals as previously employed, namely 3, 6, and 9 hours. All possible care was taken to prevent contamination of the material, but some sporadic contamination was unavoidable. As the grinding progressed, a sample

was taken after each interval, the sample being removed from directly beneath the pestle. This was placed in a sterile weighing flask and weighed. One-tenth gm. of the sample was diluted to 9 cc., giving a dilution of 1:100. From this, other dilutions up to 1 to 10^6 were made, and poured plates were arranged from these dilutions. A sample from the original bouillon culture mixed in the same proportion with the diatomaceous earth, without grinding, was plated out at similar concentrations. Duplicate cultures were made in every case. The result of these experiments indicated that even after grinding the spores of *Bacillus subtilis* for only 3 hours very few remained viable, an average of 32 per culture at a dilution of 1:100, at which dilution the control showed innumerable colonies. After 6 hours of grinding the viable spores averaged 2 per culture, and no greater dilution yielded any colonies whatsoever. After 9 hours a single colony appeared at a dilution of 1:100, and no colonies at greater dilutions. Grinding was therefore thoroughly efficient in killing the spores of bacteria.

We have carried out a variety of experiments on temperature relations, the effects of disinfectants, the action of light, etc., without securing any results that indicate unusual peculiarities of the mosaic. *In vitro* studies of the mosaic agency have likewise failed thus far to give any evidence of change in the culture solutions indicative of the activity of living organisms. In addition to these lines of research we have also undertaken extensive experiments, beginning in the winter of 1921–22, in the filtration of bacteria, with the idea of determining the capacity of such organisms to pass filters when apparently the spore or cell sizes were greater than the diameters of the pores, or lacunae, of the filters employed. These experiments have yielded results of striking interest, and in time they will be published separately. As bearing on the particular problem in hand, however, no application of the investigation seems possible, both because of the inactivity of the mosaic "virus" and the lack of evidence of any stage of the latter of microscopic dimensions.

In endeavoring to arrive at something more concrete than the mere name "virus" to explain the general nature of the mosaic disease agency, we need to recall many facts bearing upon somewhat related phenomena. From the investigations of Lindstrom

('18) the inheritance of a number of chlorophyll types is shown to be strictly Mendelian. These types involve various degrees of striping and cases in which the chlorophyll is almost or entirely suppressed, with the production of white, virescent, or yellow seedlings. Passing from these normally inherited color characteristics to those which are infectious, such cases as those of the variegated *Abutilon* and the striped *Ligustrum*, worked upon by Baur ('06, '07, '08), come up for consideration. In these cases, it will be recalled, there is a characteristic pattern of color, but there is no noticeable tissue modification. Transmission is by grafting only.

The types just discussed, without graft-infection experiments to demonstrate their peculiarities, would be considered "normal" variations. The infectiousness, however, is precisely that which was found by Erwin Smith to prevail in peach yellows. "Peach yellows" is, in part, a chlorotic disease, but it gradually leads to severe injury and ultimately death of the peach tree. The disease is not transmitted by pollen nor, so far as known, by seed, but a diseased scion will convey the disease in time to a healthy stock. This disease has been considered by many to possess a highly infectious nature, but of this infectious character the senior writer has been wholly unable to find any authentic proof. Statements indicating that it may "sweep an orchard in a few years," when followed up are found to be equally as well explained by the possibility that all the stock came from a single nursery at the same time. Scions from the same tree may have been employed. This disease, moreover, is rather closely localized in a narrow climatic zone. The claims of sporadic appearance of the disease in regions far south of Michigan and Delaware have in very few, if any, cases been adequately verified, especially since the water-shoots arising in clusters from severely headed-back or winter-injured trees possess many characteristics of yellows, clearly recognized, however, as water-shoots by the expert.

One should include in the graft-transmissible forms reference to the recent work of Blakeslee ('21), in which a graft-infectious disease of *Datura* resembling a vegetative mutation is discussed and its behavior in heredity clearly set forth. This disease has

been known as the *Quercina* form. It is not artificially transferable except by grafting, but certain other species of *Solanaceae* are susceptible through grafting. It is transmitted by seed to about 79 per cent of the offspring when pollinated with normal plants.

The case of the curly-top of sugar-beets, which is generally assumed to be related to mosaic disease, is peculiar in that no infection by diseased juice can occur until the juice has passed into the body of *Eutettix tenella*, in which it must remain a definite time interval, or incubation period, before being infectious to beets.

In this case not even grafting has been successful according to the more recent reports. In still another category with respect to infectiousness may be included the case of mosaic in sugar-cane, poke-weed, and other plants in which insect transfer is the more effective method yet found.

The well-known cases in tobacco, bean, cucurbit, and other plants wherein the transfer of juice from diseased to healthy plants, whether by aphids or by needle prick, is sufficient to reproduce the disease,—these are more closely related, as to infectiousness, to ordinary bacterial or other parasitic diseases. Detailed experiments by us confirm the view that the virus of tobacco and of related mosaic diseases do not pass readily, if at all, through uninjured surfaces. We have tested this by spraying the diseased juice on the leaves, also by placing the diseased juice in glass cells sealed to leaf surfaces for 24 hours or more. Under such conditions the virus is practically inert.

It is suggestive that in the tobacco mosaic, the tomato mosaic, and many others, the gametes do not seem to possess the virus; at least the embryo arising from the fused gametes is not diseased, while the seed-coats are. It is conceivable that the reduction division is concerned in the elimination of the disease, a possibility which, if established, would be significant. The case of the bean is, however, an apparent exception, though the possibility of infection after early embryonic development is not excluded.

Time prevents a more complete discussion of the bearing of these facts, but the trend of the evidence seems to indicate that

we have here a group of viruses which, apart from the cell, are as inactive as any colloidal particle lacking that correlated organization which is characteristic of cell life. Within the cell such a virus possesses unusual activity, obviously. So far as resistance to environmental conditions is concerned, we have to admit frankly that there may be no great difference between a living cell, and enzyme, and many types of biocolloids, but, on the whole, the mosaic virus behaves as if it were a biocolloid, yet one endowed with the power of reproduction. Now it has been frequently suggested in the literature that all these discussions as to the nature of a virus are unnecessary, since we may just as well take the easier, simpler view, and call a virus an ultramicroscopic organism. The facts are just a little out of line, if viewed in their broadest aspects; and the fascination is to go on and perhaps ultimately get a satisfactory explanation, or arrive at what may be an acceptable theory.

We cannot forget that important contributions have been made almost within the year. The d'Herelle phenomenon is itself a remarkable discovery. Here is a filterable body—call it what you like—appearing in the excretions of dysentery, which, placed in contact with the bacterial culture, is lethal to the culture; and at the same time the body propagates itself.

Again, if all viruses are minute bacteria, why are there no analogues of such microorganisms as saprophytes? Why are there none in butter, in milk, in soil, in fermentation phenomena of one type or another? While some "indications" of the existence of filterable organisms in such environments have been reported, it must be admitted that all changes in such substrates have been related to organisms that are not ultramicroscopic, and no such parallel in nature has been clearly demonstrated so far as I am aware. There are, of course, many diseases induced by extremely small microorganisms but the question is: Have we not already reached the point where our technique may always make evident some stage of such organisms? Are any truly ultramicroscopic organisms culturable? With agencies of the mosaic virus type we have made no progress, possibly because progress is not attainable by culture methods and by microscopic vision.

In respect to size relations some pertinent questions also arise. Is it, for example, possible that a protoplasmic particle may be as small, in small diameter, as a hemoglobin particle, remembering that the former must carry the properties of surface and central plasma and of nucleoplasm—indeed of all the characteristics of an individual? This particular question does not seem to us to be affected by any consideration of the magnitude of the long diameter of the individual. Such an individual could not presumably penetrate cell walls, and its rapid spread through the tissues would be dependent upon bridging protoplasmic fibrils between the cells.

If one is compelled to admit the existence of an organism of the size relations above referred to, it would seem necessary with the data at hand to conceive of it as a flagellum-like creature with perhaps a temporary hook-up of molecules or colloidal particles, conceivably with no true bordering membrane and no restricted endometabolism. The supposition that the organism might be of an extremely fluid nature would perhaps be equally unsatisfying.

Taking into consideration all the facts, we cannot avoid the impression, tentatively, that the causal agency in mosaic disease may be, in any particular case, a sometime product of the host cell; not a simple product such as an enzyme, but a particle of chromatin or of some structure with a definite heredity, a gene perhaps, that has, so to speak, revolted from the shackles of coordination, and being endowed with a capacity to reproduce itself, continues to produce disturbance and "stimulation" in its path, but its path is only the living cell.

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CITRIC ACID AS A SOURCE OF CARBON FOR CERTAIN CITRUS FRUIT-DESTROYING FUNGI[1]

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Introduction

The work reported here was undertaken with the idea that the fungi which rot citrus fruits probably show some peculiar metabolic adaptations to life in such an acid environment as that furnished by the citrus fruits in general and particularly by lemons. In the progress of the work, however, the available methods, especially those for the quantitative determination of citric acid, were found to be so unsatisfactory that it was deemed advisable to spend considerable time in studying possible methods and their application to the routine work of physiological experimentation. In the first part of this paper, therefore, considerable space is devoted to the methods utilized in this research as well as to some notes on the chemistry and occurrence of citric acid. In the execution of the physiological side of the work the utilization of citric acid as a source of carbon for fungi is the special phase considered, and only passing attention is given to that other important phase, the production of acid.

It is not the intention to propose the idea that citric acid tolerance or utilization is the primary factor in the invasion of citrus fruits by fungi; in fact, due to the structure of these fruits, it is probable that a number of parasitic fungi which cannot grow in a synthetic medium as acid as the expressed juice of lemons or grapefruit are still able to rot these fruits with comparative ease. From a microscopic examination of numerous rotted fruits and from the laboratory experimentation, the idea has been gleaned that in the primary infection and rotting of citrus fruits the ability to hydrolyze cellulose, and not tolerance of citric acid, is likely

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University and submitted as a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy in the Henry Shaw School of Botany of Washington University.

to be one of the deciding factors. Nevertheless, for those fungi which cause the ultimate destructive decay which releases the acids contained in the juice sacs of the fruit, there must be metabolic adjustment to life in an extremely acid medium.

Discussion of Citric Acid

Citric acid (oxy-tricarballylic acid) is a tricarboxylic acid with one substituted hydroxyl group, of the structure:

$$\begin{array}{l} CH_2.COOH \\ | \\ C(OH).COOH. \quad 1\ H_2O \\ | \\ CH_2.COOH \end{array}$$

It crystallizes from concentrated solutions with one molecule of water of crystallization; but if heated too much in concentrating it turns yellow and does not crystallize, probably due to the formation of other compounds. It is extremely soluble in water but less so in most of the organic solvents, such as ether, alcohol, and chloroform. It dissociates in 3 stages, and Blasdale ('18) gives a dissociation constant of 8×10^{-4} for the first stage. Davis, Oakes, and Salisbury ('23) point out, however, that in titrating citric acid with alkali a curve corresponding to that for HCl is obtained instead of one corresponding to the titration curve for H_3PO_4; this would indicate that the di-basic and mono-basic salts are not sharply separated from each other in formation, as is the case in the similar salts of phosphoric acid. Citric acid does not possess an asymmetric carbon atom and consequently does not rotate the plane of polarized light. It forms 3 classes of salts and some mixed salts, but only the tri-basic salts are usually prepared.

Potassium citrate, used considerably in the work here reported, is a soluble, strongly alkaline salt, crystallizable with difficulty from water, owing to its high solubility, but more easily crystallized by shaking out with alcohol. Sodium citrate is even more soluble in water, but is easily crystallized out as fine crystals by shaking an aqueous solution of the salt with 95 per cent alcohol. The crystals form at the junction of the 2 liquids and slowly settle

out. Handbooks ordinarily give the sodium salt as crystallizing with 11 molecules of water but when it is crystallized out from alcohol as above described the percentage of water seems to be much lower. None of the salts of this acid with the heavy metals are quantitatively insoluble in water but most of them are less soluble in alcohol. The salts of Ca, Pb, and Ba are commonly used in analysis and will be discussed under the head of quantitative methods.

Citric acid is quite readily oxidized, its salts and the acid itself being oxidized in air at less than 200° C. The acid is decomposed by concentrated sulphuric acid, sulphuric and chromic acid mixtures, by $KMnO_4$ in acid solution, and by $K_2Cr_2O_7$ under the same conditions as for $KMnO_4$, but more slowly. The general products from such oxidations are CO_2, CO, acetone, acetaldehyde, acetic acid, and formic acid, depending on the oxidizing agent and the conditions of the reaction. On account of its high carbon content and the ease with which it is oxidized citric acid should be a fairly good source of carbon for those organisms capable of utilizing it.

QUALITATIVE DETECTION

The common methods of detection are based largely upon the fact that certain salts are less soluble in hot water than in cold, i. e., precipitates are formed on heating the solution and these disappear when the solution is cooled. Calcium citrate is commonly used in this test and the acid lead salt has been recommended by the Association of Official Agricultural Chemists ('07). This sort of method is likely to be misleading, however, in the presence of other salts or in the case of too great or too small a concentration of citric acid. Stahre's pentabromacetone method, depending upon the formation of a complicated compound, pentabromacetone, when citric acid is oxidized to acetonedicarbonic acid by $KMnO_4$ in the presence of Br, has been used extensively. This test is probably less sensitive, and much less satisfactory than that of Denigés ('98), which is based upon the formation of a complicated mercury compound with acetone. As this method was used in this work it will be given in detail and follows closely the instructions by Yoder ('11). Denigés' solution is prepared by dissolving 5 gr. of mercuric oxide in 20

cc. of conc. H_2SO_4 and diluting with 100 cc. of distilled water. To about 5 cc. of the solution to be tested, containing a small amount of citric acid, is added about 1 cc. of the mercury solution, the solution heated almost to boiling, and 2 per cent $KMnO_4$ added drop by drop with shaking. After a few drops have been added a white cloudy precipitate is formed if citric acid is present. If the $KMnO_4$ continues to be used up but no precipitate is formed it is likely that sugar, oxalic acid, or some other compound oxidized by permanganate solution more easily than citric acid, is present. If these compounds are present in small quantity, citric acid may be detected by continuing to add $KMnO_4$ solution slowly until they have been completely oxidized, when the $KMnO_4$ will react with the citric acid. If the interfering substances are present in considerable amount it may be necessary to precipitate the citric acid with barium acetate and 50 per cent alcohol, and after washing the precipitate, dissolve it in sulphuric acid, filter off the $BaSO_4$, and apply the test.

According to Yoder ('11), succinic, tartaric, and malic acids do not give this test, but aconitic acid does. Amberg and McClure ('17) stated that pyruvic, ita- and citraconic acids gave positive tests, but a large number of others tried, including tricarballylic, succinic, etc., did not give positive tests. Oxalic acid gave a white precipitate on addition of the reagent, and this must be filtered off before proceeding with the test.

QUANTITATIVE DETERMINATION

There is as yet no satisfactory gravimetric method for the determination of citric acid, though a number of such methods have been offered. The lead salt which is commonly used for the primary separation is very soluble in water, and Yoder ('11) points out that at least 3.6 volumes of alcohol are necessary for a quantitative precipitation of citric and malic acids, whereas 1 volume has usually been used.

For gross work citric acid may be precipitated from a neutral solution with calcium acetate or chloride, filtered hot, washed sparingly with hot water, and the precipitate weighed as calcium citrate or converted to $CaSO_4$ and weighed. This general method is valuable only for concentrated solutions, but is commonly

recommended. For the analysis of citric acid by precipitation as calcium citrate, L. and J. Gadais ('09) collected the filtrate and washings from the first precipitation, concentrated to a small volume, and reprecipitated, adding the second residue to the first. This is probably the best modification of the use of the calcium precipitation and increases the scope of the general method considerably.

Spindler ('03) pointed out that the calcium precipitation was not quantitative but dependent upon the volume of the solution, also that tri-calcium citrate, which is supposed to crystallize with 4 H_2O, loses water when dried at 100° C. Yoder ('11) gave the limits of concentration of citric acid for producing a precipitate with calcium acetate, without stirring or scratching the sides of the beaker, as more than 0.32 gm. of acid in 100 cc. of solution in the cold, and less than 0.32 gm. in 100 cc. of boiling solution. The writer obtained by this method an appreciable crystalline precipitate from 0.025 gm. of citric acid in 100 cc. of solution by autoclaving at 15 pounds for 20 minutes. The precipitates obtained at higher concentrations of citric acid were well crystallized by this method and easily filtered. By precipitating always from the same volume of solution it might be possible to make use of the precipitation in the autoclave satisfactorily. Calcium citrate is quantitatively insoluble in 50 per cent alcohol, but apparently no work has been done on the water content of the salt when precipitated by this method.

Creuse ('72) noted that the barium salt of citric acid was almost totally insoluble in alcohol of 0.908 sp. gr., and he offered a tentative formula for the precipitated compound of the general structure: $BaO_3.C_{12}H_5O_{11}.2\ H_2O$. He precipitated from a neutral solution in 63 per cent alcohol. The precipitate by this method is gelatinous but fairly heavy, and after standing over night can be filtered, washing mostly by decantation. Jörgensen ('07) offered a method for separating the barium salt of citric acid from that of malic acid, based upon the comparative insolubility of the citrate in 26 per cent alcohol and the greater solubility of the malate in that solvent. The separation is not sharp and requires considerable manipulation, depending on the relative concentrations of the 2 acids. Moreover, the precipitate is very

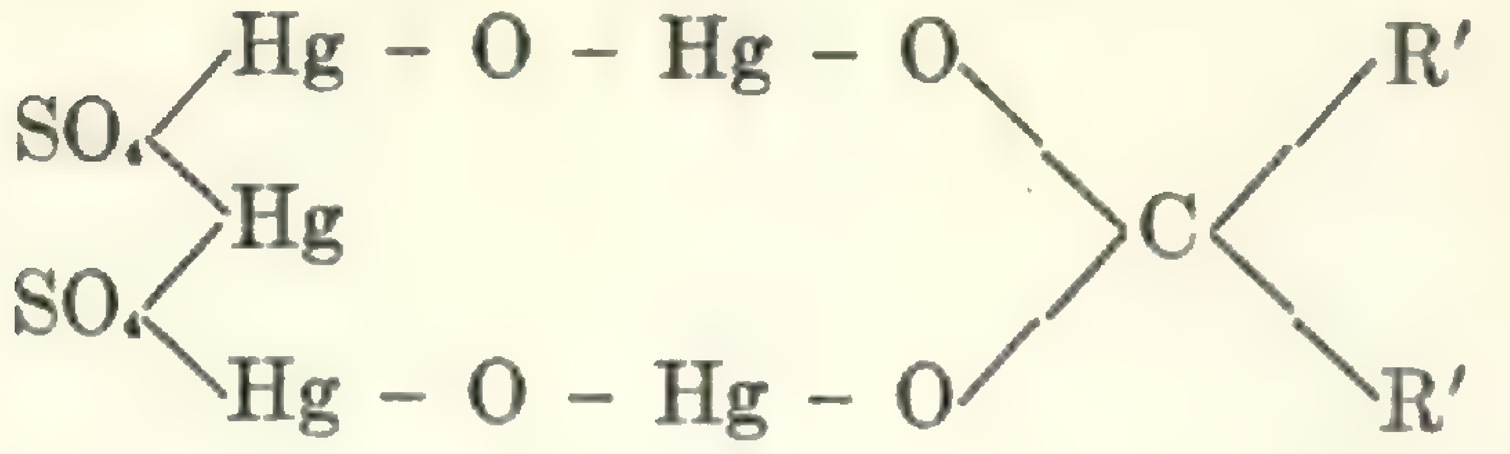
gelatinous and difficult to filter as compared with that from 63 per cent alcohol.

Several attempts have been made to base a quantitative method on the fact that $KMnO_4$ oxidizes citric acid to acetonedicarbonic acid which in turn decomposes at temperatures above 80° C. to form acetone. Jörgensen ('07) oxidized citric acid with $KMnO_4$ under various conditions but his yields of acetone were uniformly low. Fleischer ('72) obtained acetone only in the presence of mineral acid, but Jörgensen obtained it in the presence of NaOH.

Kunz ('14) modified Stahre's qualitative test and weighed the precipitate of pentabromacetone. This method has been used considerably by biochemists for determining small quantities of citric acid and has been tentatively adopted by the Association of Official Agricultural Chemists ('09). Following the lead of others Salant and Wise ('16) added Denigés' reagent to the solution to be tested, oxidized with $KMnO_4$, and weighed the mercury complex. McClure and Sauer ('22) studied both of the above methods and decided that on the whole the pentabromacetone method of Kunz was best. Both methods require tedious manipulation, are easily interfered with by substances other than citric acid, and neither gives a very good yield of the compound to be weighed.

Pratt ('12) distilled off the acetone as fast as it was formed by the interaction of citric acid and $KMnO_4$, the latter being added slowly through a separatory funnel as the reaction proceeded. The receiver flask contained Denigés' reagent. When the reaction was complete the distillate was refluxed and the precipitate weighed. Willaman ('16) modified the distillation arrangement and substituted a titration for the gravimetric determination of the acetone in the complex precipitate. This was a material improvement, since gravimetric methods are to be avoided wherever possible in the routine of physiological work where many analyses must be made.

Any method based upon the determination of the mercury-acetone complex of Denigés is necessarily empirical and is more difficult still in that the mercury-acetone complex is probably of varying as well as uncertain composition. Denigés ('98) gave for this complex the formula:



In a later publication ('98a) he stated that this formula was correct if the compound was dried at 110° C., but if dried at a lower temperature it probably had a composition represented by $[(SO_4Hg)_2.3HgO]_3.4CO.R_2$. Oppenheimer ('99) arrived at a different formula and factor for conversion to acetone and stated that there was no difference in the compound whether it was dried at 110° C. or at a lower temperature. The uncertain composition of this compound prevented a critical study of this method, since it was impossible to determine accurately the actual yield of acetone from citric acid. Theoretically 1 molecule of citric acid should yield 1 molecule of acetone, but since the rate of oxidation in some degree controlled the yield it was evident that the yield did not, in all probability, reach 100 per cent in any case. On account of this fact it was the feeling of the writer that some better method for determining the acetone might lead to a much improved method.

Pratt ('12) stated that the Messenger titration was not adapted to this method; however, Shaffer ('08) offered a method for the determination of β-oxybutyric acid based upon the oxidation of that compound to acetone by chromic acid in the presence of H_2SO_4, and the determination of acetone by the Messenger method. The interference due to the formation of volatile products from the oxidation of sugar, which were probably of an aldehyde nature, was overcome by redistillation of the first distillate, after rendering it alkaline with NaOH and adding 30 cc. of 3 per cent H_2O_2. It was at first thought that the yield was 100 per cent, but in a later paper Shaffer and Marriott ('13) showed that the yield was about 90 per cent of the theoretical and that some of the products of the oxidation of sugar were not eliminated by the redistillation, although the error from this source was negligible. Marriott ('13), studying the distillation of acetone and its determination by the Messenger method, showed that by boiling 10 minutes the acetone was completely removed to the

distillate and that if a good condenser was used the receiving flask need not be iced. Likewise, the accuracy of the Messenger titration was tested in this work and the method found to be efficient.

With this in mind the writer attempted a study of the oxidation of citric acid by permanganate, using the Messenger titration for the determination of the acetone. As only a short, straight-tube condenser was used the receivers were sometimes iced. Potassium permanganate of the concentration suggested by Pratt ('12) was used for the oxidation.

In the first experiments, using a known solution of citric acid and the conditions given by Pratt, the yields were extremely low, and although different concentrations of H_3PO_4 were used the yield never rose above 85 per cent. $K_2Cr_2O_7$ and H_2SO_4 were tried under the conditions of the Shaffer method but the yield was low. On trying H_2SO_4 with the $KMnO_4$ the yield increased to about 90 per cent and was much more constant, nor did any interference occur due to substances formed from the sulphuric acid. In using H_2SO_4 a brown precipitate was formed when the oxidation was complete and $KMnO_4$ was still added. Some of the distillations were stopped as soon as the brown color appeared, new receivers were substituted, and the distillation continued. By this method it was found that practically all the acetone yielded by the method had been collected in the first distillate, and no more than traces could be detected in the second receiver. Moreover, tests for citric acid failed when tried on the residue in the distilling flask.

As the work was being carried out with special regard to its use in culture solutions, the effect of various constituents of culture solutions was tried. The non-volatile compounds, such as sulphates and phosphates, gave no interference. Nitrates gave a substance utilizing large quantities of thiosulphate, but this compound was readily eliminated in the redistillation from alkali. Various sugars and organic acids were known to form oxidation products, and the yield of aldehyde from malic acid had been studied by Jörgensen ('07). These were studied for interference. One-half gram of malic acid was placed in each of 2 Kjeldahl flasks, and 100 cc. of water and 5 cc. of 5 N H_2SO_4 added. The

oxidation was carried out in the usual manner. One distillate was titrated directly by the Messenger method. On adding the alkali a white precipitate formed, the titration being only 4.0 cc. of thiosulphate on a blank of 23.15 cc. The second distillate was redistilled with 10 cc. of 10 per cent NaOH and 30 cc. of 3 per cent H_2O_2 and the distillate from this titrated. The titration was 23.15 with a blank of 23.25 cc. The procedure was repeated with small amounts of dextrose with the following results:

	Thiosulphate (cc.)
Single distillation	22.35
Redistilled	23.25
Redistilled	23.2
Blank	23.25

Shaffer and Marriott ('13) stated that a small amount of interfering substance, which was not eliminated by the redistillation, was formed when dextrose was oxidized by $K_2Cr_2O_7$ in the presence of H_2SO_4. Such a substance is not in evidence here, though it might be formed under certain conditions.

Using the apparatus and general procedure described under "Methods" a number of determinations were made on known solutions of citric acid, varying the time of oxidation, amount of mineral acid, volume of solution, and the amount of citric acid. Some of these results are given in table I. The results varied more than if an efficient condenser had been used. Part of the results shown were obtained from a single distillation, part were redistilled.

It may be seen that the yield with H_3PO_4 as the mineral acid was around 80 per cent and very variable. Where H_2SO_4 was used the yield varied from 87 per cent to 95 per cent, depending on the volume of the solution, the amount of mineral acid, and the period of oxidation. No one of these factors, however, is as important as maintaining the same conditions for all determinations. The amount of solution, especially, seemed to affect the yield very slightly, even when raised to 200 cc., but this made distilling more difficult. On the whole, about the best conditions indicated by the results of a large number of experiments were (1) a volume of 50 to 125 cc., (2) about 1 per cent H_2SO_4, (3)

an oxidation period of 15 minutes for 100 mgms. of citric acid, and (4) a concentration of citric acid between 75 and 125 mgms.

TABLE I

QUANTITATIVE DETERMINATIONS OF CITRIC ACID UNDER VARYING CONDITIONS

Acid added, (mgms.)	Volume, (cc.)	Interval of oxidation (minutes)	Mineral acid, (cc.)	Acid recovered (mgms.)	Yield (per cent)	Remarks
			H_3PO_4*			
100	50	28	5	82.72	82.7	
100	75	21	5	84.83	84.8	
110.77	100		3	88.2	79.6	
			H_2SO_4‡			
110.77	50		1	102.9	93.0	
110.77	50		10	97.2	88.0	
110.77	50		2	101.5	91.5	
110.77	100		2	101.6	91.6	
100	75	13	5	91.87	91.8	
100	50	11	5	93.63	93.6	
100	50	14	5	93.98	94.0	
125	50	15	5	118.45	94.9	
125	50	15	5	118.9	94.96	
75	75	8	5	65.82	87.0	
75	75	12	5	67.58	90.0	
75	50	10	5	65.64	87.0	
75	50	10	5	65.64	87.0	
75	50	12	5	67.23	89.6	
75	50	14	5	67.58	90.0	
83.07			2	74.1	89.2	
55.38			2	49.67	89.5	
110.77	100		2	102.9	92.7	Oxalate added
110.77	100		2	101.9	92.1	KNO_3, redis.
110.77	50		2	105.8	95.5	.5 gm. dextrose

* 85 per cent H_3PO_4
‡ 5 *N* H_2SO_4

Proposed Method

APPARATUS

Either the short-necked 500-cc. distilling flasks described by Willaman ('16) or the ordinary 500-cc. Kjeldahl flask is suitable for the distillation and for the receiver for the initial distillation. A spiral condenser is not necessary but is desirable. The flask

is connected to the condenser with a 2-hole rubber stopper, the second hole being used for a short drawn-out dropping tube connected with a supply bottle of permanganate as described by Willaman ('16). These tubes should be drawn down equally and the tips should be small enough to release a small drop. The distilling flask should set vertically so that the liquid from the dropping tube will not strike the neck of the flask, but drop directly into the solution.

The solutions necessary are: $KMnO_4$, 0.5 gm. per liter; H_2SO_4, conc. or preferably 5 *N* (by graduate); NaOH, saturated (60 per cent); I_2, 0.1 *N* solution; $Na_2S_2O_3$, standardized 0.1 *N* solution; soluble starch, 1 per cent solution for use as indicator.

PROCEDURE

If only small amounts of interfering substances, such as sugars and organic acids, are present, enough of the solution to be analyzed to give about 100 mgms. of citric acid should be transferred (accurate pipette) into a 500-cc. Kjeldahl flask, and sufficient water to make a volume of 125 cc., and 0.75 cc. of conc. H_2SO_4 or 4 cc. of 5 *N* acid added. This should be connected with the condenser and 25–50 cc. distilled off to remove any preformed acetone or alcohol. After this preliminary distillation the receiver should be put in place and the permanganate solution dropped at about 100 drops per minute, or at such a rate as experience shows will complete the oxidation in about 15 minutes, or longer if there are interfering substances present, such as sugars. In the Kjeldahl flask arranged as the receiver 100 cc. of cold distilled water should be used, care being taken that the receiver tube is sealed off with the water.

Distillation should continue until the brown precipitate begins to form freely. The permanganate should then be stopped and distillation continued for about 3 minutes to clear out all the acetone. To the receiving flask 5 cc. of 60 per cent NaOH and 30 cc. of 3 per cent H_2O_2 is added and distillation carried out immediately, the distillate being received in cold distilled water in a flask suitable for titrating. In distilling bring to a boil slowly and then boil vigorously 10 to 15 minutes. Fifty cc. of 0.1 *N* I_2 solution and 10 cc. of 60 per cent NaOH should then be added to the receiving

flask, this being stoppered and shaken and permitted to stand for 10 minutes. Five cc. of conc. H_2SO_4 should then be added and titrated with 0.1 *N* thiosulphate, using starch solution as an indicator. The difference between this titration and the titration of a blank on the chemicals represents citric acid (1 cc. of 0.1 *N* I_2 theoretically represents 3.5 mgms. of citric acid). To the amount of citric acid found by multiplying the number of cc. by 3.5 a correction of 6 per cent should be added if the amount is over 115 mgms., 8 per cent if from 90 to 100 mgms., and 10 per cent if less than 90 mgms. It is desirable to run known amounts of citric acid and calculate the corrections for the particular apparatus and procedure used.

Where too large amounts of interfering substances are present the citric acid must be separated by precipitation. It is not necessary, however, that this separation be complete, but only that the citric acid be precipitated quantitatively and the bulk of the interfering substances removed. Two methods are suggested as convenient and practical, the one to be used depending upon the conditions under which the work is carried out.

Method 1. Precipitation as barium citrate.—To a volume of solution equivalent to approximately 100 mgms. of citric acid a drop of phenolphthalein is added and the solution neutralized with NaOH. Just enough dilute CH_3COOH (1 to 2 per cent) is supplied to destroy the pink color and sufficient barium acetate solution to completely precipitate the citric acid, then 2 volumes of 95 per cent alcohol, and the mixture shaken. If a centrifuge provided with large tubes is available the precipitation may be carried out in one of these. The supernatant liquid should be centrifuged and decanted (the precipitate comes down rapidly when centrifuged) and the residue stirred up at the bottom of the tube with a stream from a wash bottle filled with 26 per cent alcohol and centrifuged again; repeat if necessary. The precipitate should be warmed with 5 cc. of 5 *N* H_2SO_4 and a few cc. of water and the whole washed into the Kjeldahl flask and made up to 125 cc. The flask may be boiled vigorously before connecting with the condenser to remove the alcohol, this taking the place of the preliminary distillation. The precipitated $BaSO_4$ does not interfere with the distillation and oxidation.

If it is not desirable to use the centrifuge, the alcoholic solution may be warmed over the water bath, the flask or beaker being kept covered. After a few minutes of warming the precipitate will begin to flocculate out. When the solution has almost reached the boiling point of the alcohol mixture it should be set aside over night and filtered the next day. In filtering, the precipitate should be washed as much as possible by decantation, using 26 per cent alcohol, the washing completed on the filter (2 or 3 washings are sufficient), and the filter drained to remove most of the alcohol. The filter with precipitate should be transferred to a beaker, warmed with 50 cc. of water and 3 cc. of 5 N H_2SO_4, and washed with more warm water. Continue as previously described with the preliminary distillation or boiling.

Method 2. Precipitation as calcium citrate.—The method to be followed is essentially that of L. and J. Gadais ('09), and where a large water bath or, better still, a sand bath is available the method is very convenient. A small beaker is used instead of the crucible, and the color after neutralization with NaOH is destroyed with acetic acid and the precipitation carried out with calcium acetate instead of the chloride, giving a more easily filtered precipitate. The calcium precipitate is dissolved in 50 cc. warm water and 5 cc. of 5 N H_2SO_4 and transferred to the distilling flask and the filter-paper washed with 50–75 cc. of warm H_2O.

The calcium precipitation is not quite as complete as the barium precipitation, but in some ways is much more satisfactory, the precipitate of calcium citrate being crystalline and readily filtered and washed. The disadvantage lies in the slowness of concentrating the solution, after washing, to the requisite small volume.

Where only citric acid, and no other carbonaceous substances precipitated by calcium, is present, or where the only other substances so precipitated are inorganic anions, it is both more convenient and more efficient to determine the total carbon in the precipitate by the use of the Friedeman carbon apparatus as described below. This method is also very satisfactory, in many cases, with the barium precipitation, but it must be borne in mind that other organic acids are more likely to have relatively insoluble Ba salts than Ca salts, and the precipitate must be

carefully washed with 26 per cent alcohol to free it from them and the occluded sugar.

Unless oxalic acid is present in large amounts it does no harm, but where the amount is so great as to utilize large quantities of permanganate the calcium precipitation should be used and carried out first in an excess of acetic acid. The precipitate of calcium oxalate should be filtered off, the washings and filtrate neutralized, and the regular procedure followed.

It is quite possible that the autoclave may be satisfactorily made use of in connection with the calcium precipitation. A number of experiments were tried with precipitations in varying amounts of solution, 25, 50, and 100 cc., and it was found that the amount of citric acid remaining in the solution was approximately proportional to the volume. Whether this would vary in the presence of additional substances has not been determined.

OCCURRENCE OF CITRIC ACID

Citric acid is commonly known as the constituent acid of citrus fruits. Numerous writers have given figures for the acid content of these fruits, but the figures usually represent titrations of total acid, calculated as citric acid, as is the case in most of the work done on the acidity of fruit juices. However, in the case of citrus fruits the percentage of acid is so high and the presence of citric acid so well known that these figures are fairly accurate. It is probable, however, that other acids occur in small quantities in fruits of the citrus type.

Colby ('92) gave figures for the analyses of California oranges and lemons covering the crops for 2 or 3 years. The figures in table II, taken from his work, give the percentage of total sugar and acid, calculated as citric, in the juice of certain varieties.

Chace, Wilson and Church ('21) stated that California lemons contained 3 to 4 per cent of citric acid in the whole fruit (including rind). Gray and Ryan ('21), in some work on the effects of various sprays on oranges, gave some figures indicating from 0.7 to 1.5 per cent of citric acid in normal, unsprayed oranges. Collison ('13) gave figures on Florida oranges and grapefruit of various varieties. According to these, the good marketable oranges varied from 0.35 to a little more than 1.0 per cent of citric acid

and sour stock fruit had a considerably higher acidity (the acid being calculated as the anhydrous acid and not that with the usual 1 molecule of water of crystallization). Grapefruit ran from 0.8 to 1.61 per cent acidity. The total sugar figures averaged slightly lower than those cited by Colby ('92) for California fruit. All the above figures were calculated from simple titrations.

TABLE II

ACID AND SUGAR CONTENT OF CALIFORNIA ORANGES AND LEMONS

Variety	Average for	Per cent citric acid	Per cent total sugar
Navel oranges	3 yrs.	.96	10.66
Seedling oranges	2 yrs.	1.29	12.04
Mediterranean Sweet (orange)	3 yrs.	1.28	9.30
Lemons	1 yr.	6.72–8.4	1.56–2.70

The hydrogen-ion concentration of the juice of citrus fruits, extracted from thoroughly macerated pulp, was given by Haas ('17) as P_H 2.2 for lemons, 3.0 for grapefruit, and 3.8–3.9 for oranges. Bartholomew ('23) stated that the P_H of lemons varied from 2.2 to 4.4 during the course of growth, and that the average for a large number of determinations on mature lemons was 2.31.

In discussing either the total or actual acidity of citrus fruits the structure of the fruit must be taken into account. The pulp of these fruits consists of numerous small juice sacs, each of which has a definite covering or skin consisting, according to Reed ('14), of 10–12 layers of small living cells, and inside of this covering is a pulp of broken-down cells containing acid and sugar. These sacs may be easily separated from each other under a dissecting microscope without breakage and the consequent loss of acid. Reed ('14) pointed out that while the acidity of the juice contained in the juice sacs was extremely high, indeed high enough to destroy oxidases, yet the living cells of the wall of these juice sacs contain large amounts of oxidase in an active condition. From this he drew the conclusion that the acid is retained within the juice sac by a semi-permeable membrane. The living tissues are not required, therefore, to sustain any such high acidity as is found in the extracted juice.

In examining numerous specimens of partly rotted lemons and oranges attacked by various fungi it was found that the fungus had partially digested the rag, or white pithy layer inside the rind, that it had attacked the walls of the carpels, the placentae, and in some cases the outer layers of the rind, without releasing the acid from the juice sacs themselves. Examination of some oranges in an advanced state of decay showed that the walls of the juice sacs had been attacked and the juice sacs broken down, and the same was true in less degree of lemons rotted by certain fungi. This latter condition is only the result of advanced decay, however. In many cases the examination showed the rind completely digested, but the adjacent juice sacs still intact. Even where the pulp sacs were seemingly attacked, it was only when the decay had reached a very advanced stage that the fungus could be demonstrated microscopically inside the juice sacs. Oranges should present little difficulty, as far as acidity is concerned, for decay fungi, and it is probably the case that the pulp is readily destroyed, but it is improbable that the pulp of lemons can be attacked destructively until the fungus is well established in the less acid portions.

Citric acid occurs in a large number of fruits besides those included in the citrus group, and in fact citric and malic acids make up the bulk of the acids of ripe fruits. Bigelow and Dunbar ('17) summarized the work on the acidity of fruits and added data of their own. Besides citrus fruits, most berries were found to contain citric acid, and this was especially true of cranberries, which are extremely acid. Tomatoes, cantaloupes, one variety of pear, and a number of other fruits were also found to contain citric acid. Pome and drupe fruits as a rule were found to contain malic acid.

As a product of the metabolism of fungi citric acid has been reported upon extensively by several authors. Wehmer ('93) reported on the genus *Citromyces* as containing acid producers. He obtained good yields of citric acid in the presence of $CaCO_3$. Martin ('16), using various species of the genus *Citromyces*, attempted to work out a commercially practicable method for producing citric acid by the fermentation of sugar but obtained insufficient yields. Butkewitsch ('22) studied quantitatively the

production of citric acid by *Citromyces glaber* and other species of this genus. Thom and Currie ('16) found citric acid to be produced transiently by various species of *Aspergillus.* Currie ('17), working with cultures of *Aspergillus,* was able to obtain good yields of citric acid, although Martin had previously discarded all such cultures as not producing this acid. The writer obtained citric acid from *Aspergillus* sp. and a *Penicillium* sp. on a dextrose medium.

Citric acid is not limited to the plant kingdom; it was early reported as a constituent of the milk of most mammals and of urine. Recently, quantitative studies have been carried out on the occurrence of citric acid in man. Amberg and McClure ('17) found it consistently present in urine and gave quantitative data for the amount excreted. Leake ('23) studied its occurrence in sweat under various conditions. Salant and Wise ('16) studied the physiological reaction of the animal body to varying doses of sodium citrate. While it is an excretion product in the human metabolism it actually occurs in very small quantities, however.

THE PHYSIOLOGICAL ROLE OF CITRIC ACID

As a source of carbon for fungi, citric acid, like most of the other organic acids, has received little attention. Nägeli ('80) listed it as second to tartaric acid as a source of carbon for the lower fungi. Waterman ('13) studied the use of citric acid and a number of other acids as compared with sugar, but the work on citric acid was not complete. He showed, however, that for *Aspergillus niger* this acid is a fair source of carbon. Currie ('17) suggested that citric acid might be one step in the course of the metabolism of sugar by *A. niger* and that it was used up as metabolism progressed, if the conditions were favorable. Butkewitsch ('22), working with *Citromyces glaber* and some *Penicillium*-like fungi, gave curves for the use of varying amounts of citric acid as well as for its production.

Numerous writers on pathological subjects have contributed notes on the "tolerance" of the various organic acids by fungi, but the conditions of acidity were usually not controlled and the limiting factors were more likely to be connected with the hydrogen-ion concentration than with the anion of the acid.

The work of Hemmi ('20) is a typical example of this latter type of work. Using a basic medium containing sugar and working with a large number of species of *Gloeosporium*, he studied the effect of the addition of varying amounts of organic acids. His general conclusion was that small amounts of these acids increased the growth of the fungi over that produced by the sugar alone, and larger amounts inhibited growth. The acids were added in the free state and no account taken of P_H so that the reasonable supposition would be that the depression of growth at the higher concentrations of acid was due to an unfavorable P_H. Unless the hydrogen-ion concentration is taken into account such studies give very uncertain results. The chief work of an analytical nature, therefore, has been done by workers using either *Aspergillus niger* or some of the fungi from the *Penicillium* group (including the fungi classified under the genus *Citromyces* Wehmer).

A number of earlier writers attempted to classify the products resulting from the fermentation of citric acid, and their results are outlined by Thiele ('11). From the results of these workers, using such inocula as spoiled cheese, hay decoction, etc., we find reported as fermentation products, butyric acid, acetic acid, succinic acid, ethyl alcohol, hydrogen, carbon dioxide, water, and carbonates. It is difficult to evaluate the results of the very early workers where neither culture methods nor chemical methods were well standardized, but the meager descriptions of the bulk of these experiments would point to yeasts and bacteria rather than true fungi as the organisms bringing about the fermentations. Special interest attaches itself, however, to the reported production of alcohols from citric acid, inasmuch as there was strong evidence in the present work that under certain conditions some of the fungi produced alcohols and acetic acid in the presence of citric acid, and presumably from it. Fairly recently Fitz ('78) reported alcoholic products obtained from the "spontaneous combustion" of calcium citrate. His tests gave isopropyl alcohol, a weak reaction for ethyl alcohol, and an uncertain test for succinic acid. Here again we know nothing of the actual agent of fermentation but it was probably bacterial. The difficulties in testing for such substances have no doubt hindered the work and affected the accuracy of the reports, for it is seldom that a botanist

is equipped to do careful work in organic chemistry. Currie ('17) stated that citric acid might yield oxalic acid in the course of metabolism, and my own work leads to the same conclusion, nor is it surprising that a fungus which normally produces oxalic acid from sugars should produce it from citric acid.

There seems to be a general agreement among bacteriologists that carbonates are formed by certain bacteria from the salts of organic acids and that this accounts for the increasing alkalinity of the solution. Maasen ('96) reported this finding for various bacteria and even went so far as to show how the citric and other acids were decomposed to form carbonates as end products. Ayers and Rupp ('18), Wolf ('22), and others confirm these findings. Wolf ('22) explained the reversal of reaction of *B. diphtheriae* cultures by the formation of alkali carbonates and gave figures for the amounts of CO_2 obtained from cultures of various ages as confirmatory proof. A critical examination of the data and the reactions involved would seem to indicate that this explanation might empirically delineate the situation, yet what actually happens is that the carbonates are formed as a result of the increasing alkalinity of the solution and as a consequence are a result and not a cause. Cultures of fungi were shown to produce an alkalinity as great as that produced by *B. diphtheriae* (see *Penicillium stoloniferum* and *P.* sp.) without carbonates being detectable in any appreciable amount. An inspection of the various equilibria involved may explain the situation.

H_2CO_3 dissociates in two stages according to the following two equations:

$$(1)\ H_2CO_3 \leftrightarrows H^+ + HCO_3^-$$

$$(2)\ HCO_3 \leftrightarrows H^+ + CO_3^{--}$$

Blasdale ('18) gives the dissociation constant (k) for the first equation as 3×10^{-7}, and for the second equation as 3×10^{-11}. However, H_2CO_3 is not stable in acid solution but decomposes to form H_2O and CO_2. An alkaline carbonate, such as Na_2CO_3, reacts according to the following equations when acid is added.

$$(3)\ Na_2CO_3 + HCl \leftrightarrows NaCl + NaHCO_3$$

$$(4)\ NaHCO_3 + HCl \leftrightarrows NaCl + H_2CO_3$$

$$(5)\ H_2CO_3 \leftrightarrows H_2O + CO_2$$

As the reaction proceeds to the right in equation (3) the true end point would be indicated by the dissociation constant for HCO_3 (equation (2)) and that for equation (4) by the dissociation constant for H_2CO_3, but H_2CO_3 instead of dissociating at a high acidity as H^+ and HCO_3^- decomposes to form H_2O and CO_2 and so does not accumulate in the solution as the free acid. Now if we reverse the scheme and pass CO_2 into a solution the amount of CO_2 retained by the solution is dependent entirely upon the acidity, both total and actual, of the solution. From the dissociation constant for HCO_3, 3×10^{-11}, we find that the P_H is about 5.3. On the acid side of this point the amount of CO_2 retained would be very small unless, according to Clark ('20), carbonates were used as buffers. On the alkaline side of 5.3, CO_2 would be absorbed in proportion to the total alkalinity present and carbonates would be formed (total alkalinity determined by titrating with an indicator that changes at P_H 5.3).

Consider now the situation when the salt of an organic acid such as citric acid is added to the solution and the solution left in an alkaline condition, assuming that the salt is sodium citrate. As the citrate radical is oxidized to CO_2, H_2O, etc., there is a consequent accumulation of Na ions in the solution, since the Na cannot be respired into the atmosphere and since it is exceedingly unlikely that it can all be absorbed into the organism and be neutralized. Consequently the assumption would be that in order to keep a proper degree of acidity in the protoplasmic mass it is excluded from entering in more than small quantities into the organism. The natural effect of this accumulation of the alkaline ions is the trend in the alkaline direction. Under these conditions the CO_2 respired by the bacteria is neutralized with the consequent formation of carbonates, the kind and amount depending directly on the free basic ions, the process being one of neutralization. There is certainly no valid reason for the idea of Maasen ('95) and others that carbonates *per se* are split off from the acid molecule; the obvious and direct explanation is that the CO_2 of respiration is neutralized by the basic ions resulting from the metabolism of the bacteria. Nor would it even be necessary for metallic ions to be present since some of the organic bases might

serve as well where the release of CO_2 is slow and takes place *in* the solution.

There is perhaps no ultimate difference between saying that a bacterium produces carbonates and finding carbonates formed by the neutralization of CO_2, yet there is considerable actual difference when the intimation goes with it that organic salts are "fermented" to form carbonates instead of CO_2 and H_2O. If we are to consider carbonates as products of metabolism then we must consider that H_2CO_3 and not CO_2 and H_2O are the products of respiration of most organisms and that it is only the environment which breaks up the acid into CO_2 and H_2O.

In considering the case of certain fungi using organic acids, we find these organisms growing in a medium too acid for the fixation of CO_2. As the course of active metabolism nears a close the solution rapidly becomes alkaline (see experimental results), yet not even traces of carbonates can be detected by ordinary methods. The reasons for this are probably two-fold. The fungus has used the free acid first, then the acid salts, leaving only the alkaline salts which, owing to the alkalinity of the solution, are metabolized with difficulty and the CO_2 given off by the fungous mat falls to a very low figure, and probably almost stops when even moderate alkalinity is reached. The CO_2 that continues to be given off is that resulting from the autolysis of the fungous mat but this is in large measure passed off into the air. So slowly is the CO_2 given off at this stage that the gradient through the stopper to the outer atmosphere is probably sufficient to prevent the accumulation of any considerable CO_2 tension in the atmosphere of the flask. Weakly alkaline solutions do not greedily absorb CO_2 from the air. Undoubtedly the use of a sensitive apparatus for the determination of small quantities of CO_2 and carbonates, such as that used in determinations with blood, would show considerable CO_2 evolved from these alkaline cultures upon the addition of acid in comparison with that which could be detected in the original solution. It is equally obvious that the amount of CO_2 present in the culture solution of a fungus which has ceased to consume the carbohydrate, due to the increasing alkalinity of the medium, will never equal the amount produced by bacteria growing *in* the solution and continuing active de-

struction of the acid anion, with the consequent release of basic cations to be neutralized. Instead of being the cause of increasing alkalinity, the neutralization of these basic ions by CO_2 is in all probability a means of keeping the solution from becoming even more alkaline than it ordinarily does.

The process of formation of intermediate metabolic products from citrates is very obscure. The formula of citric acid is sufficiently complex to permit of considerable adaptation to oxidation, and a few directions which such processes might follow will be indicated.

Citric and malic acids are closely allied in structure and reactions.

$$\text{Citric acid} \qquad \begin{array}{l} CH_2.COOH \\ | \\ C(OH).COOH \\ | \\ CH_2.COOH \end{array}$$

$$\text{Malic acid} \qquad \begin{array}{l} CH(OH).COOH \\ | \\ CH_2.COOH \end{array}$$

It is probable that the substituted OH group in these acids is the path of easy chemical access to the citrate and malate molecules. As was previously noted in connection with the oxidation of the various acids, succinic acid was not oxidizable either by permanganate or chromic acid, while malic (hydroxy-succinic) and tartaric (di-hydroxy-succinic) were readily oxidized by both substances, as is citric acid, which has a similar substitution. Likewise, benzoic acid and phenol are oxidizable with difficulty, whereas resorcin and phloroglucin are readily oxidized. Moreover, it is a general tenet of organic chemistry that compounds containing substituted hydroxy groups are less stable usually than the unsubstituted compound.

Sando and Bartlett ('21) pointed out that malic acid in extracted fruit juices breaks down spontaneously in the presence of toluol and chloroform to form succinic acid. Citric acid under the same conditions might be expected to form tri-carballylic acid. Both succinic and tri-carballylic acids, especially the

latter, are difficult to identify positively in small quantities. The formation of these 2 easily oxidized, hydroxy acids in such large quantities by plants, instead of the unsubstituted acids, is probably quite significant, and would class these compounds as storage products rather than as ultimate waste products.

It would be expected, in view of the ease with which citric acid is oxidized to acetonedicarbonic acid, that acetone might readily be a product of metabolism, That this may actually be the case in some instances was indicated by the fact that a distillate giving a profuse iodoform test in the cold was obtained from a culture solution of *Diplodia natalensis.*

The general indications are that CH_3COOH, C_2H_5OH, and other alcohols are produced under conditions where the O_2 supply is insufficient. The case of isopropyl alcohol and butyric acid is not well established, but it would seem probable that if one were formed the other might be formed also. However, such a splitting of the citrate molecule would apparently furnish little energy to the organism.

Miscellaneous Chemical Methods

TOTAL OR TITRATABLE ACIDITY

Sodium hydroxide and phenolphthalein were used for the titration of culture media. In part of the work the samples were aerated with CO_2-free air before titration, but comparison of aerated and unaerated samples showed so little difference that the procedure was abandoned. Titrations were carried to a strong pink color, since, according to Merck's handbook, both oxalic and citric acids are completely neutralized by this procedure. In so far as this method was applied to culture solutions it must also be noted that any KH_2PO_4 present in the solution would be titrated to K_2HPO_4.

In some of the work the amount of citrate was roughly estimated by titration with HCl, using thymol blue as indicator. Thymol blue changes from yellow to pinkish orange as the P_H changes from 1.8 to 2.0, but the end point is uncertain even when blanks of free citric acid and the indicator are used. This is especially the case when ammonium salts are present in the solution.

HYDROGEN-ION CONCENTRATION

The P_H of the solutions used was determined colorimetrically, using the Clark ('20) series of buffers and indicators.

REDUCING SUGARS

Dextrose was used throughout this work, and quantitative determinations were made by the Shaffer and Hartmann ('21) method, using the adaptation of Fehling's solution.

OXALIC ACID

For either the qualitative or quantitative determination of oxalic acid the precipitation with calcium acetate in a hot solution acidified with CH_3COOH as described by Leffmann ('17) was made use of. The precipitate from the acidified solution was dissolved in H_2SO_4 and tested qualitatively with $KMnO_4$ and MnO_2 or determined quantitatively by titration with standardized $KMnO_4$.

THE DETERMINATION OF TOTAL CARBON

It was found desirable to have a means for determining with rapidity the total carbon in the culture solutions used. This involved the oxidation of dextrose, citric acid (peptone in a few instances), and any metabolic derivatives of these substances. A number of dry combustion methods were examined, but besides being complicated and slow required too much equipment. A wet combustion method as sometimes used for carbon determinations in soil and steel seemed more feasible, while the method of Friedeman ('21) seemed to fill the need very satisfactorily. Since Friedeman's work has not been published, a brief account of the method will be included, together with data as to its application and accuracy in physiological work.[1]

Chemistry of the method.—Oxidation by chromic and sulphuric acids in the presence of H_3PO_4 is made use of in this method. The CO_2 formed when the reaction mixture is heated is aerated over into a modified Truog ('15) absorption tower and absorbed

[1] The writer is especially indebted to Mr. Friedeman for personal aid in the working up of the method and apparatus used, and for advice on the chemistry involved in its utilization in this line of work.

there with NaOH. In the original method $Ba(OH)_2$ was used to absorb the CO_2 but NaOH was found more satisfactory, being less difficult to handle in the air and forming no precipitate in the tower. $BaCl_2$ is added just before titration and the excess NaOH titrated in the presence of the precipitated carbonate according to the method of Bear and Salter ('16). The end point of the titration is very sharp but a little slow due to adsorption of the indicator by the particles of the precipitate. The difference between the titration of a blank on the chemicals and the titration of the determination represents CO_2, 1 cc. of 0.5 *N* HCl being equivalent to 3 mgms. of carbon. Concerning the technical chemistry of the method the reader is referred to Friedeman's work.

Apparatus.—A single unit of the apparatus is shown in fig. 1, and in pl. 14 is shown the equipment used, which included 4 such units. The figure and plate are largely self-explanatory. The tower (F) is made of glass tubing 25 mm. in internal diameter blown on to tubing of 5–6 mm. internal diameter. The length from "a" to "b" is 45–50 cm. and from "b" to "c" 30 cm. A perforated porcelain filter plate is placed at "b" and the tower filled about two-thirds full of glass beads (one 4-mm. perforated bead to two 3-mm. solid beads). When the joints are closed and suction applied liquid in E is drawn up over the beads in F. Carbon dioxide-free air is supplied by a soda-lime tower and the aeration tube for B should be of very small diameter, drawn down at the tip and bent as in fig. 1 to prevent the solution backing up into the tube during heating. The suction must be steady and a Richards pump was found very desirable; the bubbler G aids in keeping the suction steady, and, the addition of a drop of alkali and a drop of phenolphthalein indicates whether any CO_2 is being carried over from the absorption tower.

Reagents.—The following reagents are necessary: an oxidizing mixture, consisting of 340 gms. of chromic acid crystals (chromic trioxide) dissolved in 600 cc. of hot distilled water and diluted to 1 liter with syrupy H_3PO_4; syrupy H_3PO_4, 85 per cent, a good commercial grade; conc. H_2SO_4, 1.84 sp. gr., a good commercial grade; 0.5 *N* NaOH; 0.5 *N* HCl, standardized to be used for titration; $BaCl_2.2H_2O$, 75 gms. per 500 cc. of solution (rough

weight), 10 cc. of this solution being equivalent to 25 cc. of the 0.5 *N* NaOH.

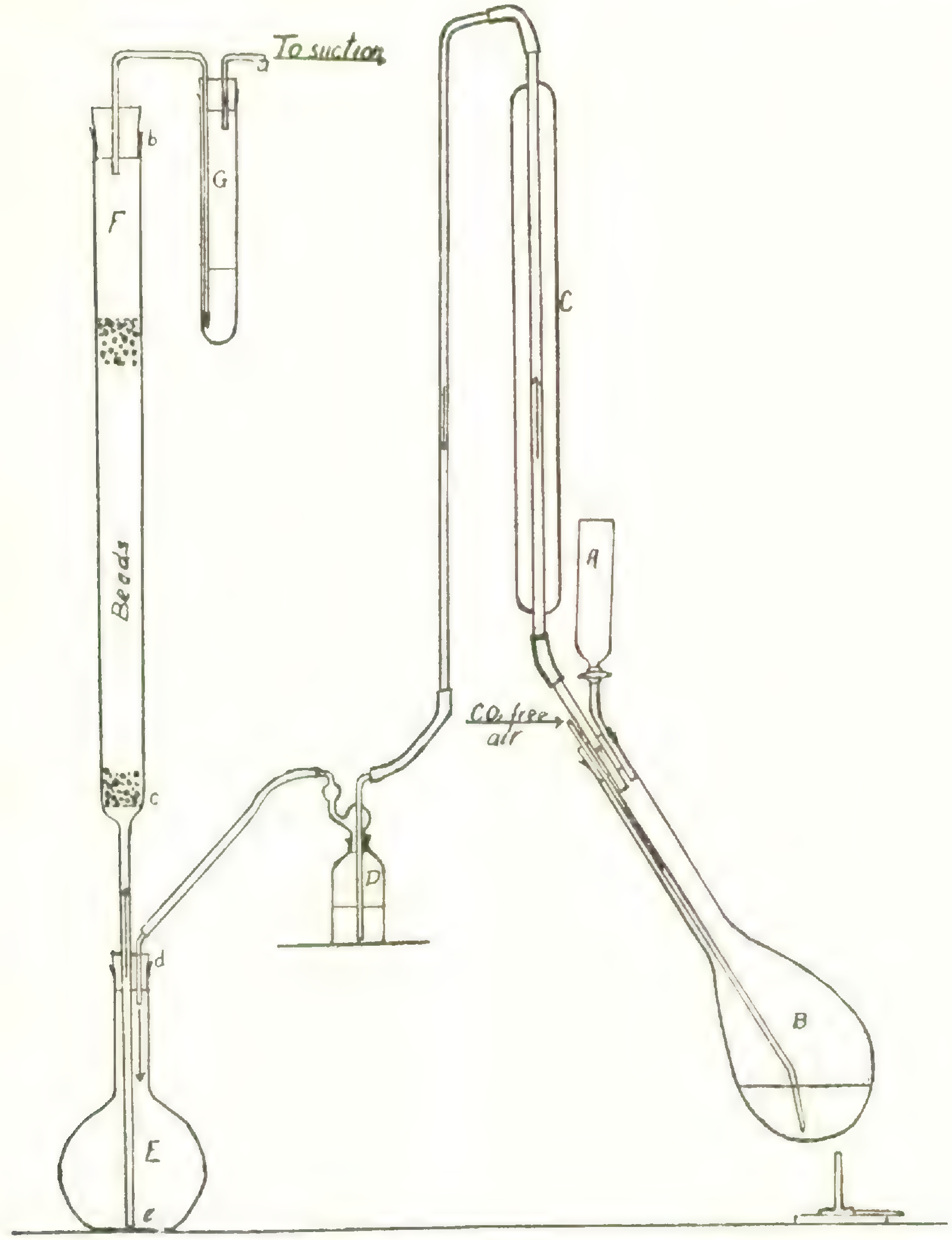


Fig. 1. Diagram of carbon analysis apparatus: *A*, separatory funnel; *B*, Kjeldahl flask; *C*, condenser; *D*, gas wash bottle; *E*, 500-cc. Florence flask; *F*, absorption tower; *G*, bubbler or trap.

Procedure.—Pipette into the Kjeldahl flask an amount of solution containing not more than 125 mgms. of carbon. Add enough distilled H_2O to make a volume of 50 cc. and a few glass

beads to prevent bumping. Attach the flask to the apparatus, make all the connections air-tight, and aerate briskly for 5 minutes to free the apparatus from CO_2. Close the pinch-cock at "a", loosen the rubber stopper at "d", and raise the tower enough to permit pipetting in 50 cc. of NaOH (25 cc. if only a small amount of carbon is present). Connect again and start aerating steadily. The solution automatically rises in the tower and covers the beads. Start the water running through the condensers. Add 10 cc. of the oxidizing solution through the separatory funnel A and follow it with 25 cc. of H_2PO_4 and 25 cc. of H_2SO_4. Heat cautiously with a low flame until the reaction mixture boils, to avoid forcing the solution back into the aeration tube. Continue boiling and aeration for 30 minutes, cut off the heat, aerate briskly for 5 minutes, then cut off the suction and raise the tower and fix it in a clamp so that it drains into the flask E. Wash the tower with small quantities of distilled water, using a total of 250 cc. and allowing a few seconds for draining after each addition. This method of washing removes practically all the NaOH to the flask E, it being less difficult to free the tower of alkali when NaOH is used than when $Ba(OH)_2$, as in the original method, since there is no precipitate of $BaCO_3$ to hold back the solution. Add 20 cc. of the $BaCl_2$ solution and 1 cc. phenolphthalein and titrate. The difference between the titration of the determination and that of a blank gives the amount of HCl equivalent to carbon.

Efficiency of the method.—Both Friedeman ('21) and Schollenberger ('16) gave data to indicate that this method of oxidation as applied to soils gave results approximating very closely those given by the usual dry combustion methods. Friedeman's ('21) figures on the combustion of cane sugar by his method indicated about 98 per cent oxidation as compared with figures by the dry combustion method. The writer was not equipped to carry out dry combustions but combustions by the Friedeman method were carried out on a number of solutions made up by weight as accurately as possible and then checked by other methods. The figures for the percentage of oxidation on duplicate determinations were as follows:

Dextrose	100 per cent
Citric acid	99.8 per cent
Oxalic acid	98.1 per cent
Malic acid	97.5 per cent
Tartaric acid	99.3 per cent

The above figures are only indicative of the general efficiency of the method, since it is very difficult to obtain some of the above acids in a pure state or to prepare accurately known solutions by ordinary methods. Some of the readily volatile substances, such as acetic acid and the lighter alcohols, are not oxidized by this method, and whether this is due to their volatilization or to their natural resistance to such an oxidation is undecided. Succinic acid is likewise unoxidized, possibly due to the formation of the stable succinic anhydride. It will be found, however, that the common fruit acids and sugars yield results of a good grade of accuracy by this method.

Experimental Work

THE FUNGI EMPLOYED

In carrying out the physiological work reported here a considerable number of fungi was used. Some of the cultures proved to be poorly adapted to culture work or too irregular in growth on artificial media and had to be abandoned, e. g., *Pythiacystis citrophthora* which failed to grow consistently in any liquid medium, and *Oospora Citri-aurantii* which was found very interesting but impracticable to use, due to the fact that no mat is formed, the growth breaking up to form a fine sediment. Some of the cultures were not obtained until the work was well under way and others failed to respond satisfactorily at various times; thus, in numerous phases of the work complete data on some of the fungi are lacking. A satisfactory culture of *Penicillium italicum* was never obtained, the culture used early in the work being so attenuated that it refused to infect oranges, and another used for a time as *P. italicum* proving later to be a different species (so identified by Dr. Thom). Nor was *P. italicum* found on rotting fruit in the St. Louis market during the winter. This was a disappointment since this fungus is un-

doubtedly an important one in the rotting of citrus fruits. The examination of a considerable amount of decayed fruit in shipments from Florida and California indicated that this fungus may not be as common in the winter as at other times, and that there are other fungi of this group with blue-green or green spores which may be at times mistaken for it. The organisms used in the work are reported upon below:

Penicillium stoloniferum Thom.—This fungus was sent by Dr. Fawcett of the Citrus Experiment Station of California. It had been isolated from rotting citrus fruit and was thought to be *P. digitatum* at the time, but it was later identified by Dr. Thom as *P. stoloniferum*. The habitat given by Thom ('10) is decaying Polypores and Boleti, but the fungus was found by the writer on decaying masses of citrus fruits. The culture was found infective to a certain degree, and it readily attacked lemons or oranges which had been partially rotted by other fungi. It probably does not constitute a primary agent in the infection of these fruits under ordinary conditions, but is of secondary importance, bringing about a final destructive rot. It grew well on most synthetic media, tolerated high acidity, used citric acid readily, and usually produced an alkaline reaction in the medium. The spores are considerably smaller than those of *P. digitatum* and the spore masses do not have the same olive-green color.

Penicillium sp.—This fungus together with the one just discussed, was sent to Dr. Thom, for it had been thought that this might be *P. italicum*, although the spores were considerably smaller than those described for that fungus. Dr. Thom stated that it was not related to *P. italicum* and that he could not name it definitely. Its growth on most media was blue-green to gray and very vigorous. It grew well on synthetic media, used citric acid readily, and usually produced an alkaline reaction in the culture medium. In general its reactions were very similar to those of *P. stoloniferum*.

Penicillium digitatum Sacc.—This fungus is well known as the cause of a destructive rot of lemons often seen in the market. It was a poor organism for cultural work since it did not grow well unless peptone was supplied as a source of nitrogen. It used citric acid, but in a fermentative way, since there was no

weight increase when this reagent was added to the medium. It is one of the commonest of the citrus fruit-rotting fungi on the market and can be easily distinguished by the olive-green color and large size of the spores.

Aspergillus sp.—This fungus was reported by Dr. Fawcett as rotting fruit at 27°, 30°, and 34° C. In spore size and the structure of the spore-bearing heads it corresponds with *A. niger*, but the spores in mass appeared first cinnamon-brown and then dark brown, and rarely if ever were dark enough to be considered black. It grew readily in most media, using the ordinary inorganic nitrogen sources. It also produced acid under certain conditions but apparently not so abundantly as the true *A. niger*. It may be a strain of *A. niger* or of a closely related species.

Diplodia natalensis Evans.—Cultures of this fungus were also furnished by Dr. Fawcett. The fungus grew well on most of the common synthetic media, forming a black, carbonaceous mat of close texture, but it did not form pycnidia readily. After growth the culture solution was dark colored, a deep red as the fungus matured, but if citric acid salts had been added this color was lessened. This fungus was originally reported from South Africa, where it caused a black rot of citrus fruits, and Fawcett ('15) later reported the fungus from Cuba, on grapefruit.

Alternaria Citri Pierce.—This fungus was also furnished by Dr. Fawcett. It was a slow-grower on most media, taking more than 20 days to come to a maximum growth. It produced spores very sparsely and did not grow at a high acidity. This fungus was originally described by Pierce ('02) as the cause of black rot of navel oranges, but the description was short and the inoculation data incomplete. Later, Patterson, Charles, and Veihmeyer ('10) reported a *Stemphylium* isolated from oranges affected with black rot. No further work has been reported in connection with the pathogenicity of this organism. This fungus will be contrasted with the next one mentioned, in regard to cultural characters.

Alternaria sp.—On the local market I found lemons apparently rotting with a typical brown rot, but in attempting to isolate *Pythiacystis* from these an *Alternaria* was frequently found. Various cultures of this organism were obtained and compared with

Alternaria Citri Pierce. The cultural characters were not greatly different, and the differences might well be interpreted as due to the development of the fungus in a different environment.

The spores of this *Alternaria* were consistently larger than those from the culture of *Alternaria Citri* and yet the spore sizes of both fell inside the limits prescribed by Pierce ('02). A brief comparison of the cultural characteristics of these two cultures is given below and checked against the description by Pierce ('02).

Spores.—The spores of the culture of *A. Citri* used were few in number, and within the limits of 11–22 × 5.5–8.2 μ in a large number of spores measured from various transfers. The spores exhibited 1–3 cross-walls, were light brown to dark brown, and in chains of 2 or 3. Germination was slow and the percentage of germination low. The spores of *Alternaria* sp. were numerous and within the limits 23.3–35.7 × 8.2–13.2 μ. There were 1–7 cells divided off by longitudinal as well as transverse walls, dark brown in color, and when viewed in position in a Petri dish culture were seen to be in long branching chains. These spores germinated readily in 24 hours at P_H 3.0, in 31 hours at P_H 2.7, and a few germinated at P_H 2.5. Pierce ('02) gave the dimensions of spores as 10–22 × 8–15 to 25–40 × 15–25 μ, 3–6-septate, dark olive-brown, and 3–6-catenulate. These limits would include both of the cultures used by the writer.

Cultural characteristics.—*A. Citri* was a slow-growing species, not doing well on inorganic nitrogen sources. On solid media (agar) it formed a nearly circular colony showing marked radiate growth with little or no aerial mycelium. The colony appeared black on both sides except for the white, growing edge. In liquid media the mat was white, growing *in* the solution. *Alternaria* sp. was a profuse grower as compared with the *A. Citri* culture used. On agar plates the culture was black when viewed on the reverse side, with a narrow white edge, and it was decidedly zonate. The upper surface was covered with white to gray aerial mycelium. Under such conditions spores were sometimes produced profusely and at other times almost not at all. The amount of aerial growth was likewise irregular, weather conditions, i. e., temperature and humidity, apparently being the deciding factors. Older cultures produced fewer spores, indicating that the failure of the *A. Citri* culture might be due to too long a period in culture. In liquid media the mat tended to become pinkish, where there was a good supply of carbohydrate, and later dark. The mat grew *in* the solution.

Pathogenicity.—When inoculated into ripe, sterile lemons *A. Citri* produced, in some instances, a small rotted area limited to the rag, but in some of these rotted areas there were signs of contamination. On inoculation, by cutting into the rind and inserting mycelium, *Alternaria* sp. produced a definite rot limited largely to the rag and

carpel walls which were completely digested. On the surface there was a dark central area surrounded by a brown area shading to pink at the outer edges. This fungus also attacked the damp cotton upon which the sterilized lemons had been placed and digested it rapidly in spite of the fact that it had only been wet with sterile, distilled water.

Cultures of these fungi were sent to Mrs. Patterson, of the Office of Pathological Collections, Bureau of Plant Industry, and examined there by Miss Jenkins, who was of the opinion that they might represent different species. Subsequently it was learned that the various California species of *Alternaria* were being studied at the Citrus Experiment Station, Riverside, so no further identification work was carried out.

Phomopsis Citri Fawcett.—This culture was furnished by Dr. Fawcett, while later cultures were easily isolated from rotting oranges from Florida. The fungus did not grow well in most liquid media as it grew *in* the solution instead of forming a mat on the surface. Due to this habit of growth, it probably obtained insufficient oxygen in liquid culture, this being indicated by the results of some of the culture work. This fungus is the cause of a large amount of the rotting of oranges shipped from the Florida district. It did not grow at high acidity nor did it rot lemons.

Sclerotinia Libertiana Fuckel.—Sclerotia of this organism were obtained from Professor Horne of the University of California Agricultural Experiment Station. The sclerotia germinated readily, but this fungus was found to be very erratic in culture. In the presence of peptone it usually grew well in liquid media, but with inorganic nitrogen the growth tended to be scant and the formation of sclerotia occurred very early in the period of growth. This is the cause of the so-called "cottony rot" of lemons which occurs chiefly in the packed crates of fruit.

In order to facilitate the making of tables and the discussion of results the organisms used will usually be referred to by number as follows:

2. *Penicillium stoloniferum*	7. *Alternaria Citri*
3. *Penicillium* sp.	9. *Aspergillus* sp.
4. *Diplodia natalensis*	11. *Sclerotinia Libertiana*
5. *Phomopsis Citri*	14. *Alternaria* sp.
6. *Oospora Citri-aurantii*	16. *Penicillium digitatum.*

Cultural methods.—In carrying out the succeeding work the

culture solution used, with one exception, was based on a solution developed at this laboratory by Dr. Duggar.[1] The final concentrations of chemicals were as follows: M/4 dextrose, M/5 KNO_3, M/20 KH_2PO_4, M/100 $MgSO_4$ and a trace of $FePO_4$. For obtaining these dilutions the following stock solutions were used: M/2 dextrose, M/1 KNO_3, M/4 KH_2PO_4, M/10 $MgSO_4$, and M/1000 $FePO_4$. For 50 cc. of medium the following amounts of these solutions were used: 25 cc. dextrose, 10 cc. KNO_3, 10 cc. KH_2PO_4, 5 cc. $MgSO_4$, and 6 drops $FePO_4$. In some instances M/1 NH_4NO_3 or a peptone solution containing 8.8 gms. per liter and considered as M/1 for nitrogen was used instead of the KNO_3 solution. Two sizes of flasks were used, 300 cc. and 100 cc., 50 cc. of medium being used in the larger and 25 cc. in the smaller. Pyrex flasks, frequently cleaned with chromic acid cleaning solution, were used almost entirely. Care was taken at all times to have the glassware scrupulously clean.

Inoculations were made into liquid culture media by the use of spore suspensions where spores were produced. Where no spores were produced the fungus was grown in plate culture on potato agar, and squares about 3 mm. in dimensions, cut around the periphery of the colony, were used for inoculation. In one series of experiments the cultures were kept at 20° and 30° C. but for the most part they were kept at 25° C. The latter temperature is probably very near the optimum for most of the fungi used, and it seemed the most suitable temperature as far as the entire group was concerned. For determining the amount of growth, the fungous mat was filtered off on a filter-paper which had been dried to a constant weight at 103° C., weighed, and labelled. The mats were weighed after a similar drying. Weights were determined to milligrams as rapidly as possible to prevent absorption of moisture while on the balance.

The relation of acidity to growth.—In order to gain an idea of the limiting hydrogen-ion concentration for the growth of the fungi used some germination and growth tests were made. In a preliminary way spores of *Penicillium stoloniferum* and *P.* sp. were tried in a citric acid solution containing no other nutrients.

[1] A paper embracing the work from which this was taken will appear in a subsequent number of the Annals.

Spores were placed in hanging drops in Van Tieghem cells, using 1, 5, and 10 per cent citric acid and checks of distilled water. In 48 hours the distilled water controls of the *Penicillium* sp. had germinated, while the *P. stoloniferum* spores had germinated strongly and showed growth and branching in the 1 per cent citric acid and only slight germination in the distilled water. In the 5 and 10 per cent citric acid none of the spores of either fungus germinated. Previous experience had indicated that these 2 fungi were probably the only ones which would germinate in free citric acid.

For further study 2 solutions were made up as follows:

Solution I.—

M/1 KNO_3	100 cc.
M/4 KH_2PO_4	100 cc.
M/10 $MgSO_4$	50 cc.
Citric acid	141 gms.
Dextrose	20 gms.
M/1000 $FePO_4$	8 cc.

H_2O (distilled) to make 1 liter.

Solution II.—

KOH, 112.5 gms. per liter.

Solution I was approximately 2 *N* acid, and Solution II was 2 *N* alkali and consequently suitable for adjusting the reaction of Solution I. A titration curve was constructed by placing 5 cc. of Solution I in each of several test-tubes and adding varying amounts of Solution II and enough distilled water to make a total volume of 10 cc. and determining the P_H of the solution. By this procedure Solution I, which furnished all the nutrients, was diluted one-half. The results of this procedure are as follows:

Alkali added (cc.)	0.0	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.4	1.5	2.0	2.5
P_H	1.7	2.2	2.4	2.5	2.6	2.7	2.8	2.9	3.0	3.1	3.2	3.3	3.8	4.1

Autoclaving these solutions failed to change the P_H appreciably. Proceeding with these data, solutions of varying P_H were made up for use in germination and growth tests.

The germination of the spores of some of the fungi was tried by means of the hanging-drop method, following the general

procedure of Webb ('21) and the solution just described. Solutions with a P_H of 3.0, 2.7, 2.5, and 2.2 were tried with checks of sterilized, distilled water. One slide with 2 rings was used for the control and 3 slides with 2 rings each for the solution. The spores were mixed with a glass rod in a few drops of the solution on a clean slide until a suspension of proper strength had been attained and then were transferred to the cover slip. All slides were incubated at 25° C. Where possible 3 counts were made of each drop and the results averaged; in some cases the germination was so heavy and rapid that it could only be estimated. A few notes were made concerning the nature of the germination where it seemed to be especially significant. The results of these tests are given in table III.

TABLE III

PERCENTAGE OF GERMINATION AT P_H 3.0, 2.7, AND 2.5

Culture	Period (hours)	H-ion concentration			Check
		3.0	2.7	2.5	
14	24	24, 25, 35	44, 36, 25	None	67, 77
7	24	None	None		Few
9	24	90 (av.)	57, 40, 95	50*	100
9	48			Drops overgrown	
2	24	44, 75	———	———	0, 71
2	36		90, 50, 75	———	
3	24	87, 1, 25	Occasional	———	23
3	48		25, 50, 25	Occasional	25

* 50 per cent where spores were bunched, lower elsewhere.

Culture 9 gave such strong germination that it was difficult to estimate the percentage, the germination being much better where the spores were grouped together. At P_H 2.5 about 50 per cent of the bunched spores had germinated in 24 hours, while practically none of the isolated spores had germinated. This may have been due to the collective action of the bunched spores on the P_H of the surrounding solution. The results were extremely irregular, especially with the *Penicillium* spp. One circle might give almost 100 per cent germination and the other circle on the same slide little or no germination. For P_H 2.2 a tube method

was used, since it was felt that the hanging-drop method was too uncertain and might not give an actual indication of the ability of the fungi to form a mat at any certain hydrogen-ion concentration. Likewise this tube method offered a means for studying those fungi which did not form spores.

Solutions were made up to P_H 3.0 and 2.7 and 5-cc. amounts pipetted into 6-inch test-tubes and autoclaved. Three tubes at each P_H were inoculated with each of the following fungi: Nos. 5, 6, 7, 11, 14, and 4, the inoculations, with the exception of *Oospora*, being made from agar plates. The tubes were slanted to allow more surface for development and were then incubated at 25° C. The results after incubation of 10 days are given in table IV.

TABLE IV

GROWTH IN TUBE CULTURES AT P_H 3.0 AND 2.7

No.	P_H 3.0	P_H 2.7
4	Beginning	Beginning, 2 tubes
5	Beginning	No growth
6	Good growth	Good growth
7	No growth	No growth
11	Good growth	Good growth
14	Beginning	No growth

The above procedure was repeated at P_H 2.5 and 2.2 and the results are given in table V.

TABLE V

GROWTH IN TUBE CULTURES AT P_H 2.5 AND 2.2

No.	P_H 2.5	P_H 2.2
2	Good mat	Good mat
3	Good mat	Good mat
6	Clouding	Clouding
9	Good mat	Good mat
11	Beginning	Beginning
16	Beginning, 1 tube	No growth

The foregoing data would indicate that only 3 of the cultures were capable of growing readily in a medium as acid as lemon juice and with an inorganic source of nitrogen. In the case of

Sclerotinia the situation is a little more uncertain but the fungus would probably make some growth at this acidity. It was probable also that the source of nitrogen might make some difference and that when an organic nitrogenous compound, such as a protein, was available growth might occur at higher acidity. For this reason the reaction of the fungi to extracted orange and lemon juice was tried.

Oranges were peeled, the juice pressed from the pulp, the pulp wet with distilled water and pressed again, and this pressing added to the first. Six hundred and fifty cc. of juice were extracted from 10 oranges in this way, filtered through cotton, and pipetted in 25-cc. amounts into 120-cc. flasks. The P_H of the juice was 3.8. All the fungi used grew well on this juice. The extracted pulp was put in flasks after being washed until tasteless, a little distilled water added and autoclaved. The P_H of the last washings was 4.4. These flasks were inoculated and the fungi grew exceedingly well. The rind was minced up and put in flasks with a little distilled water and sterilized. Organisms 2, 3, and 7 grew slowly but eventually covered the rind completely, slowly dissolving the rag. Numbers 4, 5, 6, and 9 grew very rapidly, quickly covered all the pieces, and brought on a destructive, decomposition.

A lemon-juice extract was made as with the oranges, and for one set of flasks the juice was diluted with an equal amount of water (Solution I), the P_H being 2.5. For a second set the juice was diluted with an equal volume of distilled water to which had been added 25 gms. of Bacto dextrose per 300 cc. (Solution II), the P_H being 2.5. For a third batch, 100 cc. of 0.48 N KOH was added to 300 cc. of the diluted juice, making the acidity P_H 3.9 (Solution III). These solutions were pipetted into 120-cc. flasks, 25 cc. per flask, sterilized, and inoculated with organisms 2, 3, 4, 5, 6, 7, and 9. At the end of 10 days these were taken down, and the titer and the weight of the mat determined. For the results see table VI.

From the culture work reported above the fungi would seem to fall roughly into two groups: those that grow fairly well at a comparatively high acidity (P_H 2.0–3.0), comprising organisms 2, 3, 6, 9, and possibly 11; and a group of those not growing at

such high acidity, comprising organisms 4, 5, 7, 14, and 16. Of these 2 groups of fungi, Nos. 4, 11, and 14 varied considerably and were almost intermediate between the 2 groups. In the case of culture 4 the nitrogen source would seem to be of considerable importance in determining the limiting acidity. This

TABLE VI

GROWTH OF FUNGI ON LEMON-JUICE DECOCTION

Organism	Solution no.	P_H	Cc. N/20 KOH per 10 cc.	Wgt. of mat (mgms.)
	I	2.4	32.0	
	II	2.4	34.9	
Check	III	3.9	28.0	
2	I	3.2	20.0	125
	II	2.6	28.3	164
	III	5.0	7.2	170
3	I	2.4	32.1	No growth
	II	2.4	34.0	79
	III	4.2	16.9	137
4	I	2.6	23.4	36
	II	2.6	22.6	160
	III	4.2	12.0	179
5	I	2.4	27.0	No growth
	II	2.4	27.8	No growth
	III	3.9	16.8	155
7	I	2.5	24.3	No growth
	II	2.4	30.7	No growth
	III	4.2	18.2	77
9	I	2.5	24.1	29
	II	2.4	25.5	54
	III	4.1	23.8	180

division constitutes one which could be made by separating the cultures according to parasitism. Organisms 2, 3, and 9 are little more than saprophytes and are fungi which are secondary in importance as far as the rotting of fruit is concerned. Such fungi as *Phomopsis Citri* and *Alternaria Citri*, however, are very specialized rot fungi, attacking practically uninjured fruit.

A series of experiments was run to determine the general relation of citric acid to metabolism. The first series was calculated to determine what fungi could profitably use citric acid without any other source of carbohydrate being present. The culture solution previously described was made up, but a mixture of citric acid and potassium citrate was substituted for the dextrose, 8 gms. of citric acid and 12.35 gms. of potassium citrate (equivalent to 8 gms. of citric acid) per 800 cc. of solution. This solution was used in 100-cc. flasks, and the cultures were in triplicate. The results after 25 days of incubation at 25° C. are given in table VII. The weights of mats as given represent averages of 3.

TABLE VII

GROWTH OF FUNGI WITH CITRATE AS SOLE SOURCE OF CARBON

No.	Wgt. of mat (mgms.)	Remarks
2	55	Solution light orange-yellow, spores plentiful, mat gray.
3	71	No spores, mycelium white to gray, solution yellow.
4	No growth	
6		Growth with white sediment and clouding.
7	21	Just beginning growth.
11	12	Very slight aerial growth.

A second solution was made up, using the mineral nutrients in 1/5 the usual concentration and with 1 gm. of dextrose per liter. No citric acid was added at the start, but 200 cc. of citric acid solution were made up, using 21 gms. of citric acid, and this was sterilized in 10-cc. amounts in test-tubes, to be added to the flasks after growth had begun. This amount of citric acid, on being added to the solution in the flasks, gave 35 cc. of 3 per cent citric acid solution. Four 100-cc. flasks were prepared for each of the fungi used. After incubation for 8 days at 25° C. all of the cultures were found to be showing definite growth, and 2 of each 4 flasks inoculated with a fungus were removed to the transfer room and a tube of the citric acid solution added to each under sterile conditions. They were then returned to the incubator after agitation to mix the citric acid with the rest of the

solution. At the end of 25 days the weights of the mats were determined, and these results are given in table VIII. The weights for mats in both the blanks and the solutions to which citric acid was added represent the average of 2 mats.

TABLE VIII

GROWTH OF FUNGI WITH FREE CITRIC ACID

No.	Blank		Solution plus citric acid	
	Mat (mgms.)	Remarks	Mat (mgms.)	Remarks
2	19	Solution orange-yellow, spores grayish	167	Growth heavy, spore masses greenish
3	31	Little growth	201	Spores green, much white aerial mycelium
4	36	Complete mat formed	32	Incomplete mat
5	14		27	All growth *in* solution
7	22		32	
11	17	Solution black, some aerial growth	42	No aerial growth

The effect of nitrogen source on utilization of citric acid.—In order to test the effect of various nitrogen sources on the utilization of citric acid a stock solution was made up as follows: citric acid, 157.56 gms.; potassium citrate, 162.17 gms.; dextrose, 12.5 gms.; M/1000 $FePO_4$, 4.0 cc.; and distilled H_2O to make 1 liter.

Five cc. of the above stock solution diluted to 25 cc. gave a nutrient solution containing M/4 of the citrate and dextrose at the rate of 2.5 gms per liter. This solution had a P_H of 4.2. It was used as the source of carbon in the regular culture solution, the source of nitrogen being varied, KNO_3, NH_4NO_3, and peptone being used. It was found impossible to use $Ca(NO_3)_2$ satisfactorily due to the fact that on sterilization calcium citrate precipitated out, or if the nitrate was not added until after sterilization, as soon as the fungus grew a little the citrate began to precipitate out due to the change in P_H. The mineral nutrients were used in 2 concentrations, as originally given and 1/5 of that concentration. The amount of the solution furnishing the carbon was kept constant throughout. Blanks were run on the amount

of sugar used but without the citric acid, using KNO_3 as nitrogen source. The fungi were grown in triplicate in 100-cc. flasks, and the results are found in table IX, the weights of the mats representing averages of 3.

TABLE IX

GROWTH OF FUNGI ON CITRATE WITH VARIOUS N SOURCES

Cult. No.	Mineral nutrients, regular conc.				Mineral nutrients, $\frac{1}{5}$ conc.				Days
	KNO_3	NH_4NO_3	Peptone	Blank	KNO_3	NH_4NO_3	Peptone	Blank	
2	201	189	307	7	167	199	225		20
3	171	258	370	52	209	208	213		20
16	91	130	162	29	47	111	168		33
4	280	353	351	60	297	347	325	22	22
11	210	244	296	58	160	171	155	15	22
7	188	149	343	42	210	134	273	13	45
5	62	68	61	32	44	75	151	14	28
9	322	301	443	37	280	275	295	16	20

From these experiments we may draw certain general conclusions to be used in future culture work. None of the fungi would make any rapid or luxuriant growth with the citrate ion as the sole source of carbon but some would utilize it if a little sugar was allowed at the start. Organisms 2, 3, 9, and probably 6, grew very well on the free citric acid, unneutralized, after they were once started with a small amount of sugar (1 gm. per liter was sufficient). Organisms 4, 5, 7, 11, and probably 16 (judging from later work), would not make more than slight use of unneutralized citric acid even after being given a good start with sugar, the free citric acid being probably lethal to the mycelium. In most cases peptone was the best source of nitrogen in connection with the citrate radical, although there was no considerable advantage over KNO_3 or NH_4NO_3 in many cases, and with *Diplodia* NH_4NO_3 would seem to make a more favorable nitrogen source than either peptone or KNO_3. In interpreting the results where various sources of nitrogen were used it is to be noted that *Phomopsis Citri* and *Penicillium digitatum* showed very little use of citric acid and that *Alternaria Citri* showed little use in the presence of KNO_3 and NH_4NO_3. None of these results are to be taken as final in any sense. No culture work which only

accounts for growth over certain fixed periods is absolutely comparative—only growth curves plotted from frequent determinations can be truly comparative.

Use of varying amounts of citric acid.—Using peptone as a source of nitrogen a large series was run, using varying percentages of citric acid-potassium citrate mixture. The solution used was based on analyses of oranges and lemons given by various authors. Using M/10 $MgSO_4$ and M/5 KH_2PO_4, the following solution was used: KH_2PO_4, 300 cc.; $MgSO_4$, 30 cc.; peptone, 30 gms.; dextrose, 15 gms.; M/1000 $FePO_4$, trace; distilled H_2O to make 1 liter. This was diluted to make 1½ liters, this dilution allowing for the addition of the citric acid mixture. The citric acid solution contained 0.25 gm. per cc. and the P_H was adjusted by the use of a solution of potassium citrate equivalent to the acid solution in citrate ion. Using varying amounts of the citric acid and potassium citrate solution, titration curves were made for solutions containing 2½ and 5 per cent of the citrate radical; and another series of curves was constructed for the titration of the culture solution with citric and hydrochloric acids. From these data solutions were made up to P_H 3.0 and 4.5, using 3 different concentrations of acid, that is, 2½ and 5 per cent citric acid (citrate radical), just sufficient citric acid to obtain the desired P_H, and a check solution with just enough HCl to obtain the desired hydrogen-ion concentration. The fungi were grown in triplicate in 2 temperatures, that is, 18–20° C. and 30° C. At the end of varying periods, depending on the speed with which the fungus grew, the weights of the mats and the P_H of the culture solution were determined. The concentration of the dextrose was rather small (10 gms. per liter), and the fungi were dependent chiefly upon the citrate-citric acid mixture for carbonaceous material. The results are given in table x, the weights representing averages of triplicate cultures.

In considering the results shown in table x certain factors in regard to the solution must be kept in mind. The weights for the HCl blanks probably do not represent the maximum weight attained, the maximum in most instances probably coming before the cultures were taken down. This loss of weight by autolysis was probably not very great in any case except in that of *Sclero-*

tinia, the mats for this fungus being left for a long period due to slow growth in the citrate solution. The increasing amounts

TABLE X

GROWTH OF FUNGI IN MGMS. ON VARYING PERCENTAGES OF CITRATE

Acidifying agent	20° C.				30° C.			
	P_H 3.0		P_H 4.5		P_H 3.0		P_H 4.5	
	P_H	Mat	P_H	Mat	P_H	Mat	P_H	Mat
Culture 2–12 days								
HCl	7.6	189	7.0	168	3.8	25	6.0	156
Cit. Ac.	6.9	218	7.3	203	3.4	—	4.5	—
2½% cit.	6.8	330	6.9	332	3.3	135	4.6	185
5% cit.	6.7	444	7.0	447	3.4	45	6.6	353
Culture 3–12 days								
HCl	7.3	225	7.2	231	3.9	—	6.9	178
Cit. Ac.	?	257	7.5	223	3.7	47	4.5	—
2½% cit.	?	393	8.1	365	3.0	—	4.7	78
5% cit.	5.7	544	7.9	475	3.1	59	4.7	162
Culture 9–12 days								
HCl	6.4	220	4.6	211	6.2	178	6.4	171
Cit. Ac.	5.7	359	5.7	224	6.5	235	6.4	210
2½% cit.	6.2	429	5.9	414	6.1	345	6.4	303
5% cit.	4.6	649	6.6	511	6.1	487	6.4	404
Culture 4–12 days								
HCl	4.0	210	7.2	305	7.5	256	7.6	246
Cit. Ac.	3.6	221	7.2	305	7.5	280	7.9	228
2½% cit.	3.3	92	5.0	316	4.6	303	7.4	328
5% cit.	3.2	—	4.7	329	3.0	—	7.4	396
Culture 7–18 days								
HCl	3.0	24	8.1	168	3.0	—	7.4	127
Cit. Ac.	3.4	16	7.0	215	3.0	—	8.1	170
2½% cit.	3.2	—	4.4	134	3.0	—	6.4	334
5% cit.	3.0	—	4.5	102	3.0	—	4.4	397
Culture 11–43 days								
HCl	6.6	90	6.0	99	4.4	157	4.6	33
Cit. Ac.	6.6	176	6.3	104	5.0	183	5.2	51
2½% cit.	6.3	212	6.6	193	4.5	171	5.1	128
5% cit.	3.1	300	4.4	146	3.2	—	4.8	288

of citrate radical in the solutions represent more than an increasing amount of carbohydrate material and increasing osmotic pressure;

in addition they represent an extremely strong buffer. Thus if the optimum for a fungus is P_H 4.5 and it is inoculated into a medium adjusted to P_H 3.0 the amount of buffering in the solution is likely to be a factor in the end result. On the other hand, if the H-ion concentration of the solution into which the fungus is inoculated is near the optimum of that for the fungus, then the highly buffered solution will maintain an optimum condition longer against the production of acid or alkali than will the unbuffered solution. In the case of organism No. 4 at P_H 3.0, the growth became less as the amount of citrate radical was increased, when incubated at 20° C., but at 30° C. the growth increased up to the 2½ per cent concentration, the fungus not growing at all in the 5 per cent concentration. That this was most likely a case of buffer action maintaining an unfavorable reaction is indicated by the final hydrogen-ion concentrations. At 30° C. this concentration varied from P_H 7.5 to P_H 3.0. At the former concentration the fungus grew, while at the latter it had not grown and the P_H was unchanged. That the amount of citrate radical is not in itself the factor that decides whether the fungus will or will not grow is shown at P_H 4.5 where the growth increased progressively with the amount of the citrate radical added. The results for several of the fungi indicated clearly that the ability of a fungus to grow at a certain P_H is not based entirely on the hydrogen-ion concentration and nutrients but that temperature and other environmental conditions are vital factors to be considered as well as the ease with which the P_H of the solution can be shifted. Organism No. 2 grew well in all of the solutions at 20° C. and about equally well at P_H 3.0 and 4.5, but at 30° C. it grew very little at P_H 3.0 and well at 4.5. Organisms 4 and 9 were the only ones that grew satisfactorily at 30° C.

The use of free citric acid.—Further data on the ability of the various fungi to utilize free citric acid were obtained by using a fermentative method. The fungi were grown in 300-cc. flasks until they had formed a substantial mat but had not yet reached the peak of growth. The solutions to be tried—a sugar solution and a citric acid solution—containing a small amount of mineral nutrients, were sterilized in 50-cc. amounts in 120-cc. flasks. After the mat was formed the solution was poured off under aseptic

conditions, 50 cc. of sterile distilled water were added to the mat, allowed to stand 5 minutes, then poured off and added to the culture solution first removed. The 50 cc. of the sterile solution in the 120-cc. flask was then poured into the 300-cc. flask with the culture. All manipulations were carried out as carefully as possible, and care was taken to wet the top of the mat as little as possible when adding the water and the new solution. Pyrex flasks were used for the mats so that the necks could be flamed freely to avoid contamination. Three solutions containing 3 different nitrogen compounds, KNO_3, NH_4NO_3, and $Ca(NO_3)_2$, were used for comparison. The P_H of the combined solution and washings was determined and the solution used for this determination returned to the flask and the titer of the solution determined, using N/10 NaOH and phenolphthalein.

The solution used for the growth of the fungi was the regular solution diluted, the following concentrations being used: dextrose, 25 gms.; KNO_3, 40 cc.; KH_2PO_4, 40 cc.; $MgSO_4$, 20 cc.; $FePO_4$, trace; H_2O to make 1 liter.

The sugar solution tried fermentatively contained: dextrose, 25 gms.; N source (KNO_3, NH_4NO_3, or $Ca(NO_3)_2$), 25 cc.; KH_2PO_4, 10 cc.; $MgSO_4$, 5 cc.; $FePO_4$, trace; H_2O to make 1 liter. This solution was adjusted to an acidity of P_H 2.5 with HCl before sterilization. The citric acid solution was the same as the sugar solution except that 25 gms. of citric acid were substituted for the dextrose. The schedule of changes to which each of the 3 mats of each fungus was subjected was as follows:

Mat 1. KNO_3*-dex. → KNO_3-cit. → $Ca(NO_3)_2$-dex. → $Ca(NO_3)_2$-cit.

Mat 2. NH_4-dex. → NH_4-cit. → KNO_3-dex. → KNO_3-cit.

Mat 3. ——— → $Ca(NO_3)_2$-cit. → NH_4-dex. → NH_4-cit.

It must be remembered that there must always be a slight difference between the blank of the solution and the solution removed from the fungous mat, even if there has been no reaction on the part of the fungus, since it is manifestly impossible to wash the mat completely free of the solution without unduly exposing it to contamination.

* The solutions will be differentiated as far as the nitrogen source is concerned by indicating the cation of the compound used, i. e., "Ca-dex" for "$Ca(NO_3)_2$-dextrose."

The data concerning the solution in which the mats were grown is given in table xi. It will be noted that 1 flask of each 3 was permitted to grow an additional period of 6 days, the original inoculations all being made on the same date.

TABLE XI

HYDROGEN-ION CONCENTRATION AND TITER OF CULTURE SOLUTION AFTER GROWTH OF FUNGOUS MATS

Organism	P_H	Cc. N/10 NaOH	Period (days)
Blank	4.4	5.15	8*
4	6.5	2.35	8*
4	6.8	1.4	14
9	1.8	57.5	8
9	2.1	37.7	14
2	7.5	1.05	9
2	7.8	0.0	15
3	5.4	6.25	9
3	6.7	1.2	15
7	6.4	3.05	10
7	6.8	2.0	16
11	5.0	5.12	10
11	5.0	8.5	16

* Data for the shorter periods represent averages of two cultures, for the longer periods one culture.

The results of the fermentation of the sugar solutions are given in table xii. These solutions were run in 2 separate series. The first series included KNO_3 and NH_4NO_3 as nitrogen sources and the second series, which followed after the first citric acid series, contained all 3 nitrogen sources. The solutions are listed according to the cation of the nitrogen-containing compound.

The results obtained when using the citric acid solution are given in table xiii. The 9-day series was run after the first sugar series and the 5-day series after the second sugar series.

A survey of the data in tables xi, xii, and xiii shows considerable variation among the fungi used, and these variations indicate in some measure the kind of reaction favorable to such a fungus. *Diplodia* produced more acid from sugar in the presence of NH_4NO_3 than when KNO_3 and $Ca(NO_3)_2$ were used, but in the case of *Aspergillus* sp. the most acid was found when KNO_3 was used. In the presence of KNO_3 the final titration of organism

4 (table XII) was less than the titration of the blank, while in the case of organism 9 all titrations on sugar media were more than

TABLE XII

HYDROGEN-ION CONCENTRATION AND TITER OF DEXTROSE SOLUTION AFTER FERMENTATION BY VARIOUS FUNGI

Organism	Solution	P_H	Cc. N/10 NaOH	Period of fermentation (days)
Blank	Ca	2.5	4.0	
	K	2.5	4.0	
	NH_4	2.5	4.6	
2	K	6.9	1.1	6
	NH_4	2.1	9.2	6
	Ca	7.2	1.0	3
	K	6.7	0.4	3
	NH_4	2.4	4.5	3
3	K	5.2	2.3	6
	NH_4	6.5	1.7	6
	Ca	7.4	0.8	3
	K	7.2	0.3	3
	NH_4	4.4	3.2	3
4	K	6.8	0.95	6
	NH_4	2.2	7.3	6
	Ca	4.2	5.2	3
	K			
	NH_4	2.4	21.75	3
7	K	7.0	0.8	6
	NH_4	4.2	2.1	6
9	K	2.1	13.5	6
	NH_4	2.9	8.9	6
	Ca	2.7	6.6	3
	K	2.2	18.35	3
	NH_4	2.0	8.35	3
11	K	6.0	1.1	6
	NH_4	2.1	10.3	6
	Ca	6.8	0.7	3
	K			
	NH_4	2.2	11.1	3

the blank. With the exception of *Penicillium stoloniferum*, in the presence of NH_4NO_3, no titration of the culture solutions of

TABLE XIII

HYDROGEN-ION CONCENTRATION AND TITER OF CITRIC ACID SOLUTION AFTER FERMENTATION BY VARIOUS FUNGI

Organism	Solution	P_H	Cc. N/10 NaOH	Period of fermentation (days)
Blank	Ca		170.0	
	K		170.0	
	NH_4		171.0	
2	Ca	8.2	2 drops HCl	9
	K	8.2	2 drops HCl	9
	NH_4	7.6	1.05	9
	Ca	7.8	1.0	5
	K	7.9	0.9	5
	NH_4	7.5	1.1	5
3	Ca	8.2	2 drops HCl	9
	K	7.9	0.25	9
	NH_4	8.0	1 drop HCl	9
	Ca	7.4	1.3	5
	K	8.0	0.6	5
	NH_4	2.8	23.2	5
4	Ca	2.4	136.8	9
	K	2.8	134.3	9
	NH_4	2.2	145.9	9
	Ca	2.4	111.25	5
	NH_4	2.4	119.0	5
7	Ca	2.5	150.1	9
	K	2.4	145.0	9
	NH	2.4	136.75	9
	Ca*			
9	Ca	4.2	0.9	9
	K	4.4	1.75	9
	NH_4	3.7	2.15	9
	Ca	6.6	1.3	5
	K	3.8	5.9	5
	NH_4	3.4	3.3	5
11	Ca	2.8	74.4	9
	K	5.8	9.7	9
	NH_4	2.3	94.7	9
	Ca	3.6	40.3	5
	NH_4	2.6	29.4	5

* Solution not changed, no growth since last change.

a *Penicillium* or of *Alternaria Citri* was more than the blank. *Sclerotinia Libertiana* produced acid in the presence of NH_4NO_3 but not in the presence of the other nitrogen sources.

In utilizing free citric acid the 2 species of *Penicillium* and *Aspergillus* sp. were undoubtedly most efficient. On blanks of about 170 cc. of N/10 NaOH the readings for these fungi, even after the short period of 5 days, were in the neighborhood of 5 cc. of N/10 NaOH or less. It is difficult to decide whether the source of nitrogen had any effect on the destruction of the acid, the case of organism 3 over the 5-day period being the only one which gives any indications (NH_4NO_3 seemed in this case a little less efficient than the other nitrogen sources). *Alternaria Citri* failed to survive the treatment with citric acid. There was an indication that some acid was used in the 9-day period but the amount was so small that possibly the very thick and spongy mat may have held back sufficient acid in washing to account for the loss. In the case of *Sclerotinia Libertiana* and *Diplodia natalensis* the figures would indicate that there was greater use of citric acid in the second period of 5 days than in the first period of 9 days. This may be due to the increased growth of the mat, resulting in greater absorbing surface. It would hardly be safe to attribute it to "acclimatization" of the fungus to this acid environment, although this might be the case. It will be noted that in the course of changing the solutions the original solution was made up with KNO_3. The first change was to a sugar solution with NH_4NO_3 and KNO_3 as nitrogen sources, while at the next change the same nitrogen sources were retained for these 2 mats. In both cases these mats were apparently dead or nearly so when the NH_4NO_3-citric acid solution was removed, nor did the mat revive and show growth when the second sugar solution was added. Peculiarly enough, when the NH_4NO_3-citric acid mixture was added to another mat on the second round, that is, the mat which had received the $Ca(NO_3)_2$-citric acid mixture on the first round, the usage of citric acid was practically as good in the case of *Diplodia* as with the $Ca(NO_3)_2$-citric acid solution, and in the case of *Sclerotinia Libertiana* a little better. Just how such data could be properly interpreted is a question.

Having obtained from the foregoing experiments sufficient

data to give a general understanding of the reactions of the various fungi, it seemed desirable to round out the work with a careful analytical study of their reactions to citric acid. For such a study it was desirable to compare a solution which contained only a sugar as a source of carbon with one containing citric acid in addition to the sugar. Growth data covering fixed periods were considered inadequate for such a study and analyses of the culture solution were resorted to. Such analyses and the weights of the mats were taken at frequent periods during the course of growth and curves were plotted from the data so obtained. This method gave a complete outline for comparison of the 2 solutions and obviated the difficulties due to variations in the periods of growth in the 2 solutions.

The culture solution developed by Dr. Duggar and described previously in this article was utilized in this part of the work. Had there been time available for extended comparative work it is probable that a more satisfactory solution could have been found for any one of the fungi or perhaps for the entire group. However, the solution used appeared to be well adapted to the group of fungi as a whole. Wherever possible KNO_3 was used as a source of nitrogen, since it simplified the analytical work; some of the fungi, however, required peptone, and for *Diplodia* NH_4NO_3 was used. Dextrose was used as the source of carbon since it was easily determined quantitatively and had given good results previously. The citric acid used was Merck's "Reagent," and the KOH and potassium citrate Merck's "Highest Purity."

The question of what cation to use in the partial neutralization of the citric acid was a difficult one. Ammonium citrate gave growth with all the fungi as both a nitrogen and supplementary carbon source, when used with a small amount of dextrose, but the NH_4 radical complicated the solution unnecessarily and was probably not an efficient source of nitrogen for most of the fungi. Sodium would probably have been satisfactory in many instances but its exact status in relation to the growth of fungi is unsettled and, at least in some instances, it appears to be toxic. Calcium precipitates an insoluble salt with citric acid on heating, and even if $Ca(NO_3)_2$ is added to the solution after sterilization calcium citrate is precipitated out as soon as the fungus starts

growth. The determining factor in the latter precipitation is apparently the hydrogen-ion concentration of the solution. The use of potassium would seem to unbalance the solution by increasing disproportionately the amount of this cation, since KNO_3 and KH_2PO_4 were being used as inorganic nutrients. However, potassium had already been used successfully, and as the citric acid was to be only partially neutralized it seemed preferable to use potassium rather than a cation of unknown physiological reaction. In adding a large amount of citrate radical to the solution it was apparent that one solution was to have a somewhat higher osmotic pressure than the other. To compensate for such a disparity it would have been necessary either to cut down the dextrose in the solution to a small amount and to substitute sufficient citrate mixture to make up for the dextrose or to add to the dextrose solution enough of an inert buffer substance to be equivalent to the osmotic pressure of the citrate mixture. Both of these methods would involve numerous difficulties. If the first method were used the amounts of dextrose in the 2 solutions would be so widely different as to make difficult an accurate comparison of the growth in the 2 solutions even if the amount of the citric acid-potassium citrate mixture to be added could be accurately determined, and the second method is at present impossible owing to the fact that no absolutely inert (physiologically) buffer mixture for culture media is known.

Using the usual concentrations of mineral nutrients, one solution contained M/4 dextrose as a source of carbon and was designated as solution 1. This solution, where necessary, was adjusted with H_3PO_4. A second solution contained M/4 dextrose and M/4 *citrate radical* (a mixture of citric acid and potassium citrate) and was designated as solution 2. The adjustment of P_H was accomplished by varying the ratio of citric acid to potassium citrate. When only a small amount of potassium citrate was needed, neutralization with 2 *N* KOH sufficed, but where a larger amount was needed solid potassium citrate was added. The resulting solution probably contained free citric acid and a mixture of 3 potassium salts of citric acid, that is, mono-, di-, and tri-basic citrates. In so far as possible the solutions were made up in bulk and distributed to the 300-cc. flasks by means of a

50-cc. volumetric flask. This procedure insured the uniformity of the culture solution in the various flasks. All cultures were incubated at 25° C.

For each experiment with a single fungus enough flasks of solution were made up for 2 cultures to be used for each set of determinations. These determinations were made at first on each flask and the results averaged. Later the contents of the 2 flasks were mixed together and the analysis on the mixed solution taken as the average. The fungous mat was filtered off on a weighed and folded filter and washed with distilled water, the filtrate and washings being collected in a clean 200-cc. volumetric flask. The flask was then made up to the mark with distilled water and mixed. Where the mat was sufficiently coherent it was washed as much as possible in the flask before removal to the filter. The titer, P_H, reducing sugar, and total carbon were determined, and where time was available other tests were carried out. The determinations were started on the first day that appreciable growth was visible and followed up at intervals calculated to give a number of points on the curve where change was rapid and fewer where the change was slower.

Where a large amount of routine work is to be carried out, long tedious processes of analyses must be eliminated in favor of shorter and simpler procedures even at the expense of absolute accuracy. Moreover, in dealing with the growth of organisms the variations are likely to be so great as to nullify the accuracy of any single determination. In interpreting the results, likewise, it is far more desirable to have a considerable number of results indicating a continued difference than to have a single result indicating a single difference. No matter how accurate the latter might be, the variations are sufficient to make it no better than an even chance that the single result represents a variation from the normal condition. These considerations were kept in mind in interpreting the results of the experimental work, and conclusions have been drawn only from clear, consistent differences.

In plotting the curves the loss of carbonaceous matter from the solution was plotted rather than the experimental figures, that is, the successive determinations were subtracted from the blank, giving differences or losses, and these losses were plotted. These

curves compare better with the mat-weight curves, which may be run parallel to them on the same figure, and likewise represent very well the progress of the metabolic activities. On the ordinates are plotted weights in tenths of a gram and on the abscissae the time in days, the ordinates in case of the mat-weight curves representing the weight of the mat in grams. The curves represent the actual analytical work, and in order to reduce the amount of detailed material presented the tables from which these curves were plotted are omitted. Dextrose and citric acid are both plotted as carbon, dextrose on the basis of 39.978 per cent carbon, and citric acid as 34.272 per cent, these percentages being calculated from the molecular weights of anhydrous dextrose and citric acid with one molecule of water of crystallization. In the following curves besides mat weight will be found: (1) "loss of dextrose" (calculated as carbon) as determined by the Shaffer method; (2) "loss of carbon" calculated from the total carbon determinations; (3) "loss of carbon—loss of dextrose" calculated by subtracting the figures for curve (1) from the figures for curve (2) (theoretically if no oxidizable end products were formed from either dextrose or citrate, this curve would represent the loss of citrate—actually it probably roughly approximates it); and (4) a curve for loss of acidity calculated from the titrations with NaOH (the results of the titrations in N/10 NaOH were calculated as citric acid and the equivalency of carbon determined from the percentage of carbon in citric acid). This latter curve might involve several errors due to such factors as the absorption of titratable phosphates, the production of oxalic acid from the citric acid or the production of acidic substances from the dextrose, nor would it throw any light on such a situation as might be brought about by differential absorption of the anion and cation of the citrate, that is, if the anion were absorbed and the cation remained in the solution to neutralize free citric acid present in the solution the titrations would indicate a utilization of free citric acid rather than combined citrate radical.

Organism 3 (*Penicillium* sp.) and organism 9 (*Aspergillus* sp.) were used in the first series. The solutions were made up to P_H 2.5. In the curves given for these fungi each figure plotted represents the average of 2 different determinations made on separate flasks of the culture.

Penicillium sp.—In fig. 2 are given the data for solution 1 and in fig. 3 the data for solution 2. It is to be noted in connection with these curves that the time elapsing between inoculation and the beginning of chemical determinations was 5 days in the case of solution 1, but in the case of solution 2 it was 10 days. Taking

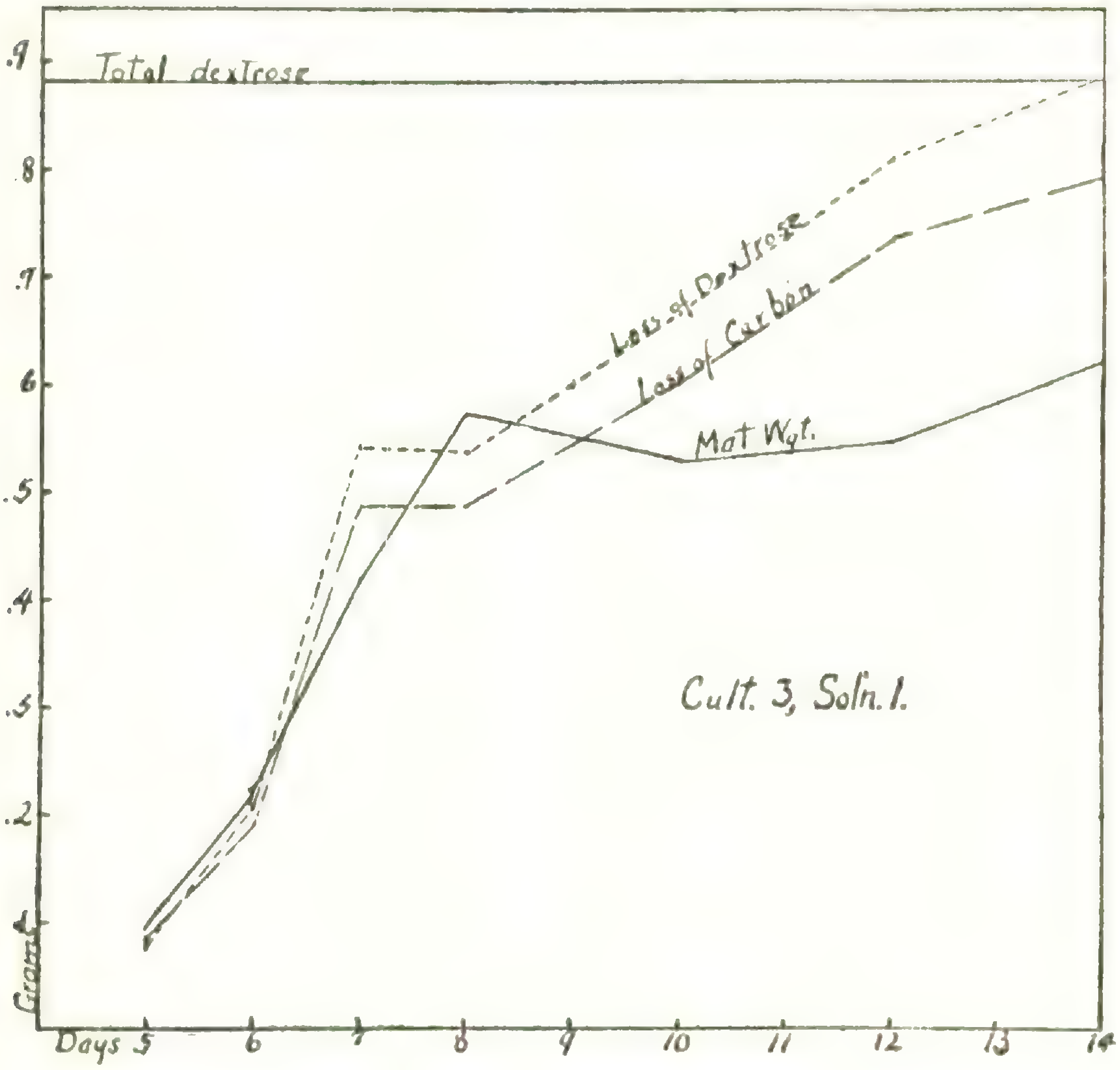


Fig. 2. *Penicillium* sp. in solution 1.

this into consideration there is to be noted a general deterrent effect upon the starting of growth in solution 2; this was probably due to the fact that the P_H of the solution was unfavorable for growth and that this situation was overcome much more readily in the slightly buffered sugar solution than in the heavily buffered solution 2.

The maximum weight of mat attained in solution 2 was not ar from twice that attained in solution 1. Apparently in solution

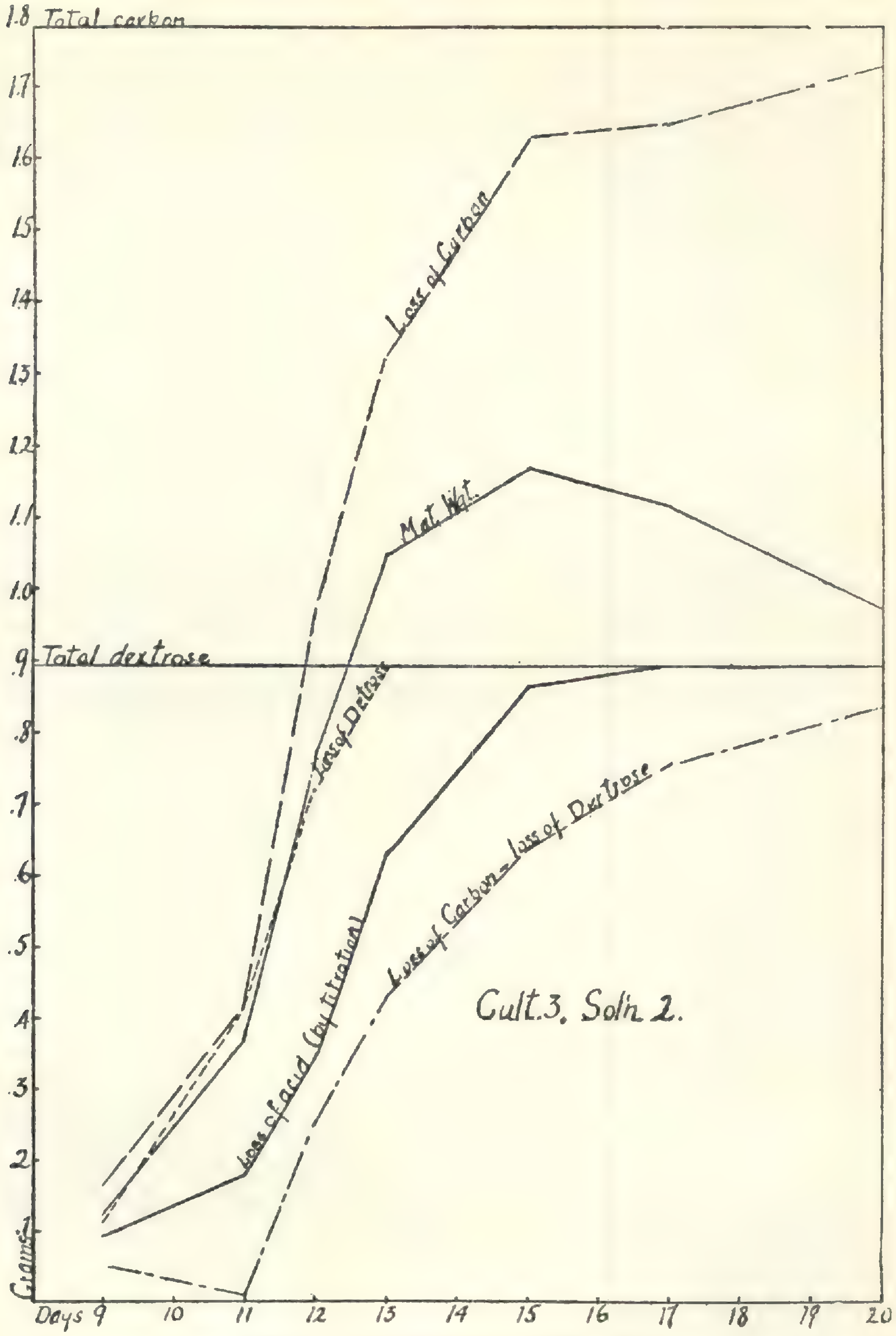


Fig. 3. *Penicillium* sp. in solution 2.

1, a point was reached at which the loss in weight due to autolysis just about balanced the increase due to the utilization of the

small amount of dextrose left in the solution. Mat weights for solution 2 showed a normal rise and fall in this respect, attaining a decided maximum and then falling off steadily.

Sugar had disappeared completely from both solutions on the fifteenth day after inoculation, but if the time is considered as beginning when good growth started, then the utilization of sugar in solution 2 was much more rapid than in solution 1. The amount of mat at the point when the sugar completely disappeared from the solution was much greater in solution 2 (about 1.5 gm.) than in solution 1 (about 0.62 gm.). This might have been due to the additional citric acid used or to the citric acid combined with a favorable effect upon the utilization of the dextrose.

The utilization of the citrate radical was remarkably complete in the case of this fungus. The amount of acid by titration had fallen to 2.1 cc. on the sixteenth day after inoculation. Beyond this point the utilization slowed up, but on the twenty-second day there was only about 0.05 gm. of carbon left in the solution, which was less than the amount remaining in solution 1 at the point when the dextrose could no longer be detected. This means that the small quantity of the citrate which was combined with potassium as the cation had disappeared. That the utilization of this combined compound was slower than that of the free acid is illustrated by the falling off in weight of the mat while the combined citrate was still being utilized. This fungus would undoubtedly be classed as a strong user of citric acid.

Aspergillus sp.—In figs. 4 and 5 are found the curves for *Aspergillus* sp. Rapid growth in the 2 solutions began about the same time and the determinations were started on the second day in both solutions. In solution 2 the maximum weight of the mat was about 0.35 gm. greater than that in solution 1, an increase of about two-fifths. This increased maximum weight was attained at about the time that the dextrose disappeared from the solution. The additional carbohydrate would seem to serve as an auxiliary to the dextrose, but in its absence it was not sufficiently available to keep up the increase of weight, since the weight commenced to decrease while the citric acid was still being used. The dextrose, however, was more rapidly used in solution 2 than in solution 1.

This may have been due to the fact that the solution was buffered at a P_H favorable for the utilization of the dextrose, and that this buffering stabilized the solution against the effect of waste products on the reaction. This is well borne out by a comparison of the data on the P_H of the solutions, since on the fourth day solution 1 had reached a reaction of P_H 3.8, and solution 2 a re-

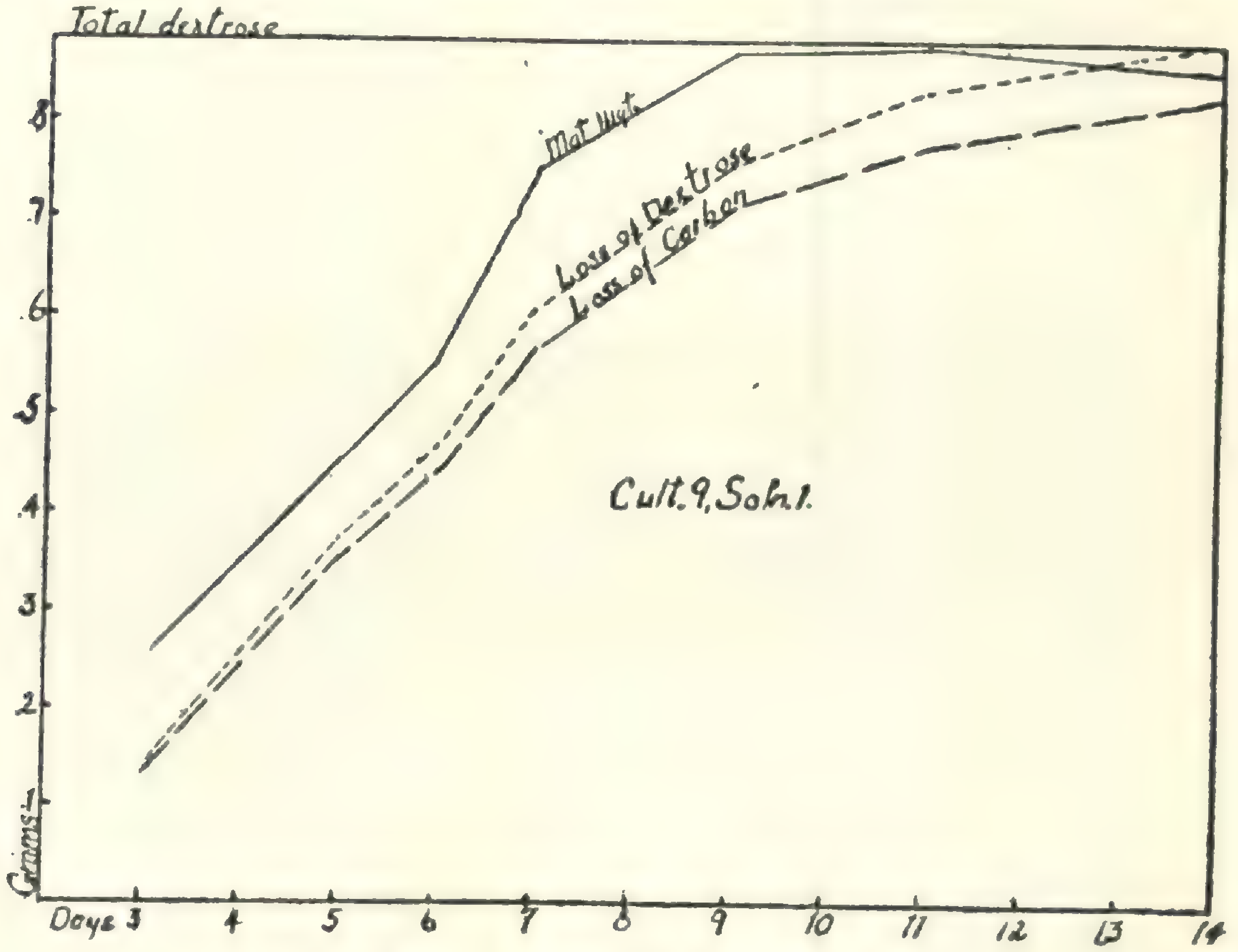


Fig. 4. *Aspergillus* sp. in soiution 1.

action of 2.6, and while from the fourth to the tenth day the reaction of solution 1 only reached P_H 3.9, solution 2 on the tenth day had a P_H of only 3.0, the progress in an alkaline direction having been very gradual. The difference was not very great but might be sufficient to account for the more rapid utilization of dextrose in solution 2.

The utilization of carbon in solution 2 did not indicate a very rapid utilization of citric acid, nor did the titration figures indicate it. Had the experiment been run longer a much larger amount of citric acid would probably have been used. The titration for the blank was 46.6 cc. of N/10 NaOH (for 25 cc. of the diluted

solution), and the titration at the time of the last determination was 26.1 cc. N/10 NaOH on the same amount of solution, indicating that less than half of the acid had been used. The difference

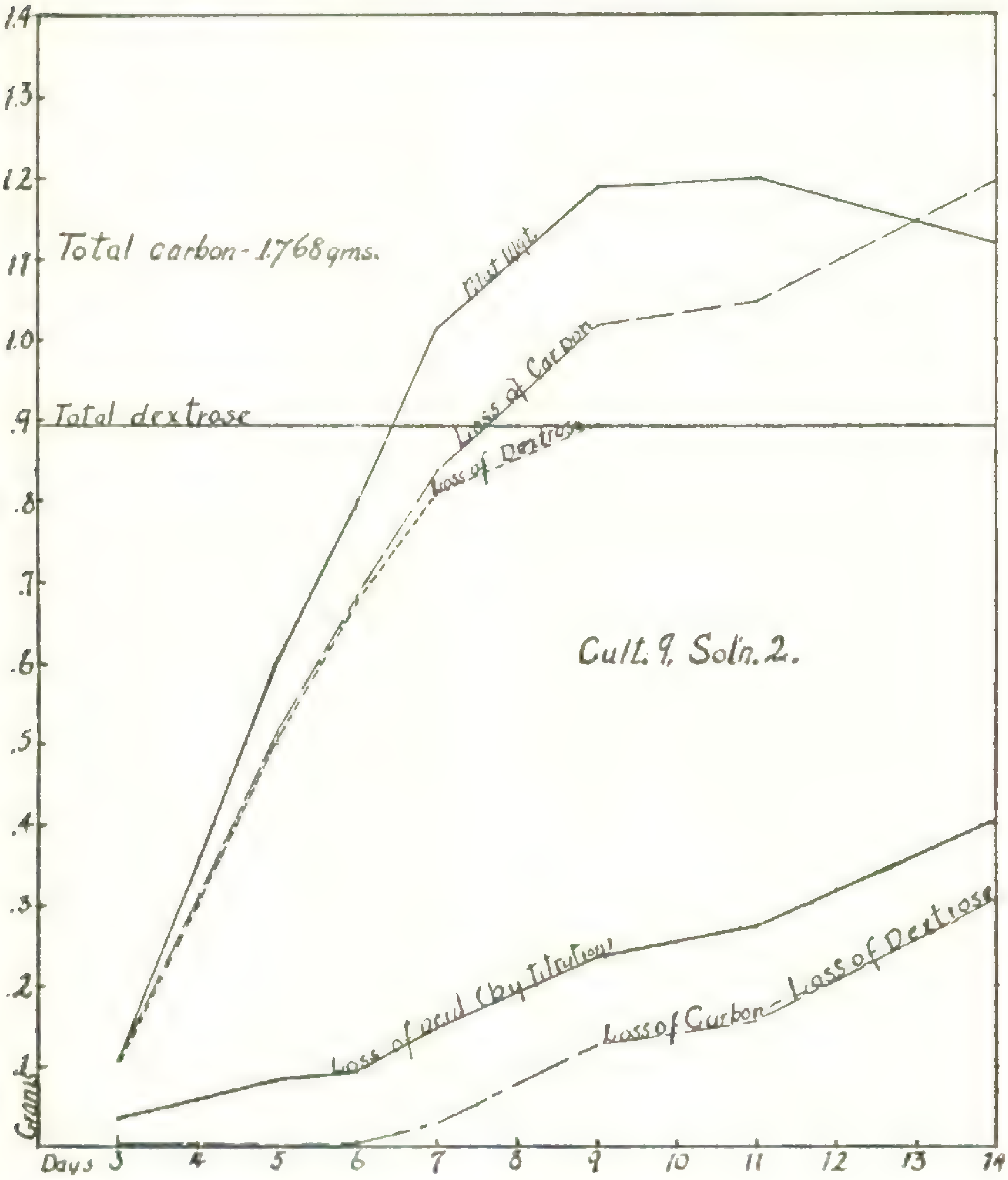


Fig. 5. *Aspergillus* sp. in solution 2.

between the total carbon-dextrose curve for cultures 9 and 3 is very marked, the rise of this curve being very rapid in the case of organism 3 and very slow in the case of organism 9.

Penicillium stoloniferum.—In figs. 6 and 7 are found the curves for the metabolism of this fungus, and the curves for the trend

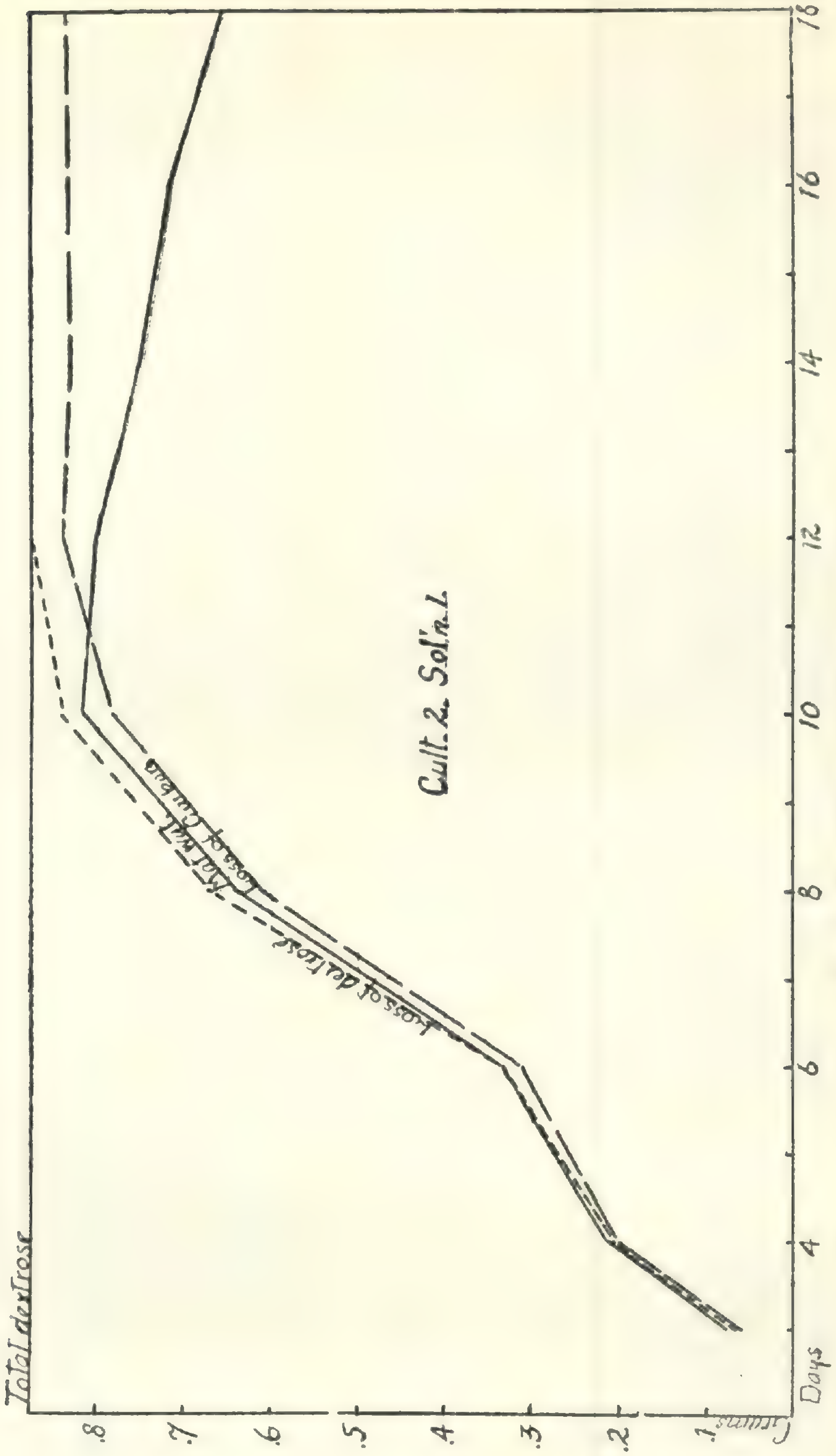


Fig. 6. *Penicillium stoloniferum* in solution 1.

of P_H for the 2 solutions will be found in fig. 8. For this fungus the solution was made slightly more alkaline than in the preceding work, solution 1 containing 20 cc. of 2 *N* H_3PO_4 and solution 2, 175 cc. of 2 *N* KOH per liter. So closely did the duplicate

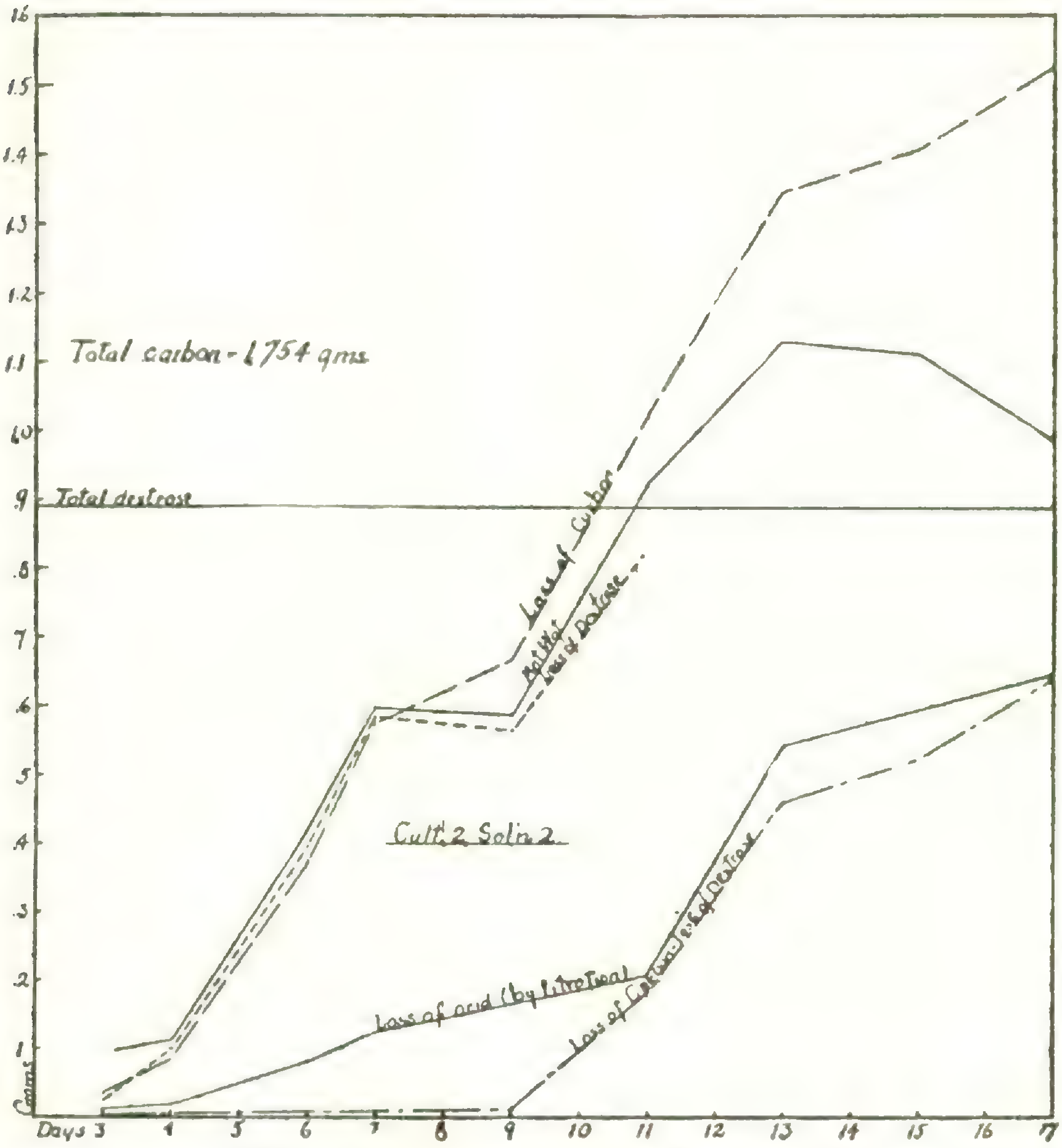


Fig. 7. *Penicillium stoloniferum* in solution 2.

mats and chemical analyses agree in the case of organisms 3 and 9, and so great was the amount of routine required that it was decided to mix the duplicate solutions in each case and run one determination on the mixed solution in all the succeeding work.

The mat from solution 2 was about two-fifths heavier than that from solution 1, as was also the case with organism 9, and the

maximum weight occurred very shortly after the dextrose disappeared. Following the maximum there was a steady decline in solution 1, but an even more rapid decline in solution 2, which is not so easily explained, especially as the indications are that citric acid was being used fairly rapidly at the same time. In the curves for solution 2 is noted a break at the fifth to seventh day, and at the same time the mats which had become rather gray-green with spores showed signs of renewed growth and the formation of tufts of white mycelium took place. This was noted at other times under similar conditions, but whether it had any significance is doubtful. However, it was peculiar that this should take place at a time when there was also a marked increase in the utilization of citric acid.

The final disappearance of dextrose from the solution was a little slower in solution 2 than in solution 1, but the mat was heavier in solution 2 at this point than that in solution 1 at the same point in the course of metabolism. This indicated a sparing of dextrose due to the presence of the citric acid, which might have been due to the buffering at a P_H somewhat unfavorable to the utilization of dextrose.

The curves for the trend of P_H (fig. 8) showed that in solution 1 there was a marked increase in alkalinity as soon as the fungus had begun to grow rapidly. In solution 2, however, as would be expected in such a strongly buffered solution, there was a long-maintained curve at nearly the original acidity, the trend of alkalinity coming very rapidly toward the end of the experiment and coordinate with it a rapid decrease in the weight of the mat. According to data obtained later, if solution 2 had been left longer a P_H approaching closely to 9.0 would have been attained. This final falling off should have been coincident with the disappearance of free citric acid and most of the acid salts from the solution.

The utilization of citric acid here was very complete and compared favorably with the utilization by *Penicillium* sp. (fig. 3). There was considerable loss of acid before the dextrose had disappeared from the solution, and although there continued to be a loss of acid after the disappearance of dextrose the mat steadily declined in weight, indicating that citric acid by itself was probably not very efficient as a source of carbon.

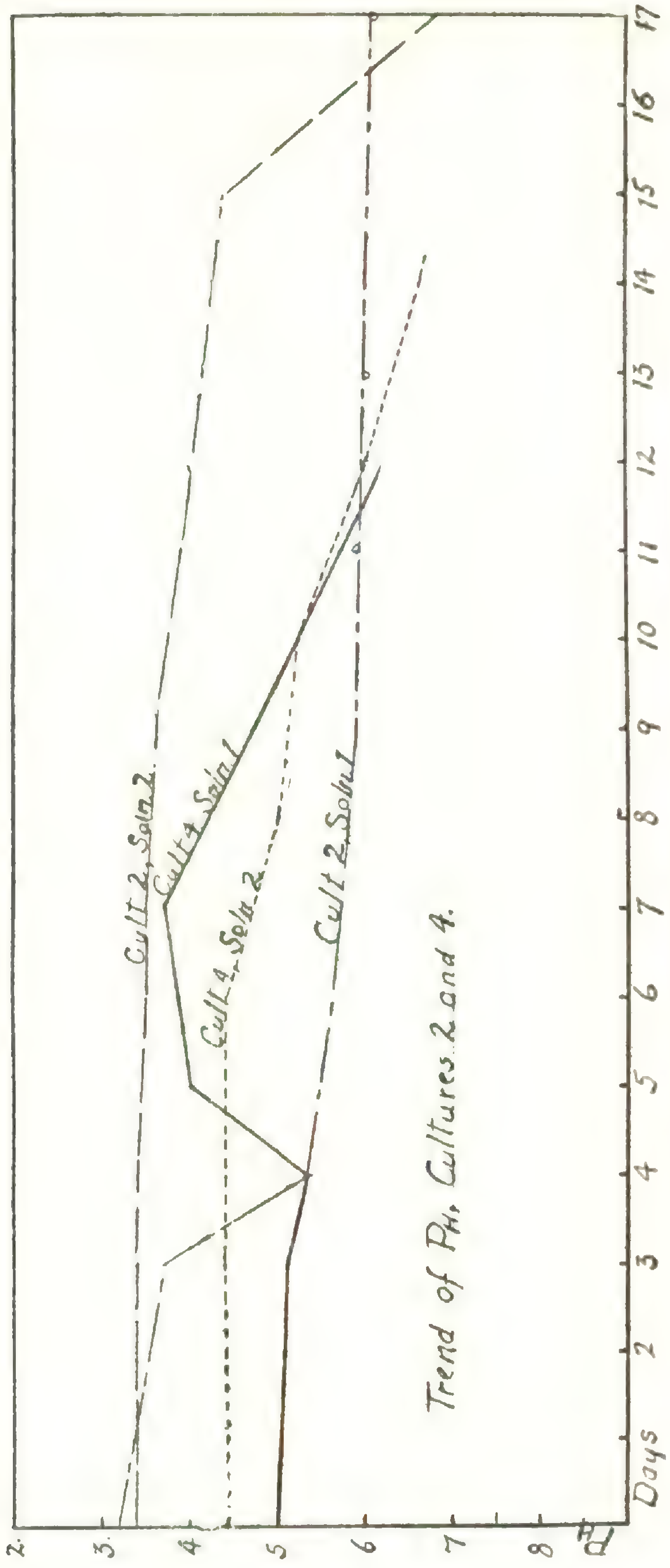


Fig. 8. Trend of P_H for *Penicillium stoloniferum* and *Diplodia natalensis*.

The curve for the "loss of carbon—loss of dextrose" remained very constant around zero until the ninth day when it started to rise very rapidly, indicating that citric acid was being utilized; as will be seen, this corresponded with the figures for the titration of the free acid.

Penicillium digitatum.—The curves for the weights of the mats are given in fig. 8, the analytical data in figs. 9 and 10. Owing to the fact that *P. digitatum* grows very little with inorganic nitrogen sources, peptone was used instead of KNO_3, an equivalent amount of the peptone solution formerly made up (see p. 245) being used. According to the figures on the carbon determinations, peptone gave about 25 per cent oxidizable carbon under the conditions of the determinations. The amount of peptone added was so small, however, as compared to the amount of carbon present that no attempt was made to remove this substance from the solution in making analyses. In the case of sugar determinations it is probable that there are small amounts of copper-reducing substances in peptone but the error is so small, and the removal of peptone before the determinations without taking out some of the sugar is so difficult, that the determinations were run without removing the peptone from the solution.

In solution 2 separate analyses were made of the citric acid in the following manner: The acid was precipitated by the barium method as previously described and the precipitate dried. The dry precipitate was dissolved in warm concentrated H_3PO_4 and transferred to the reaction flask of the carbon-determination apparatus and the carbon determined in the usual way. As soon as the H_2SO_4 was added $BaSO_4$ was thrown down, but this caused no difficulty in carrying out the determinations. The peptone gave a little interference in this procedure but this did not amount to more than 3–6 mgms. of carbon per determination of 75–100 mgms. of carbon. This was done because citric acid had been found to disappear in the preliminary cultures and little growth had resulted.

The growth curves shown in fig. 9 show that as far as the 2 solutions were concerned there was little difference in value, if the weight of mat be used as the criterion. Solution 2 produced about the same growth as solution 1, but was a little slower in

doing so and the final decline in weight was correspondingly slower. The dextrose had disappeared 2 days earlier in solution 1 than in solution 2, and if the mat curves were smoothed out there would be about that much difference between the times of attaining the maximum weight of mat in the 2 solutions. Apparently these maximum weights had been attained shortly before the dextrose was completely used up. This is uncertain, however,

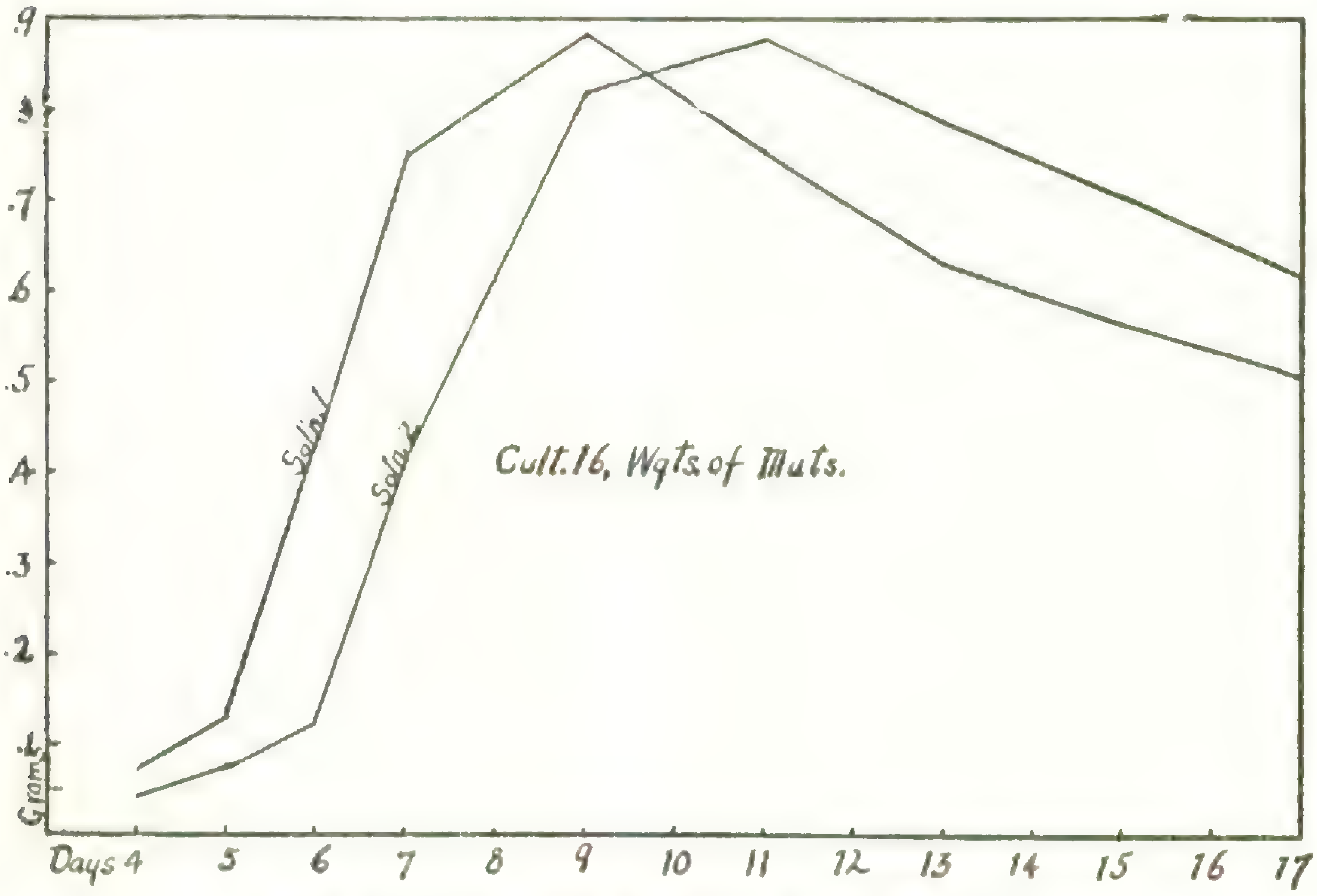


Fig. 9. *Penicillium digitatum*, weight of mats.

since in such a case as that in fig. 10 there was a slight amount of dextrose on the eighth day and none on the tenth day, but if the curve for the loss of dextrose be calculated according to the conformation of the curve previous to the eighth day, the disappearance of dextrose would be found to occur some time on the eighth day or near the beginning of the ninth day.

The presence of the peptone in the solution complicates the problem of drawing conclusions from the data obtained. The remarkable fact in connection with solution 1 (fig. 10) is that the curve for the loss of carbon seems to tally almost absolutely with that for the loss of dextrose, and would consequently make it appear that little or none of the easily oxidizable carbon in the

peptone was utilized. This was probably not the case, however, and this is further indicated by the difference between the curve for "loss of carbon—loss of dextrose" and the loss of citric acid as determined by analysis (fig. 11). The P_H of solution 1 ran from 5.5 at the beginning to 4.1 and back to 6.4 where it remained for most of the course of the experiment.

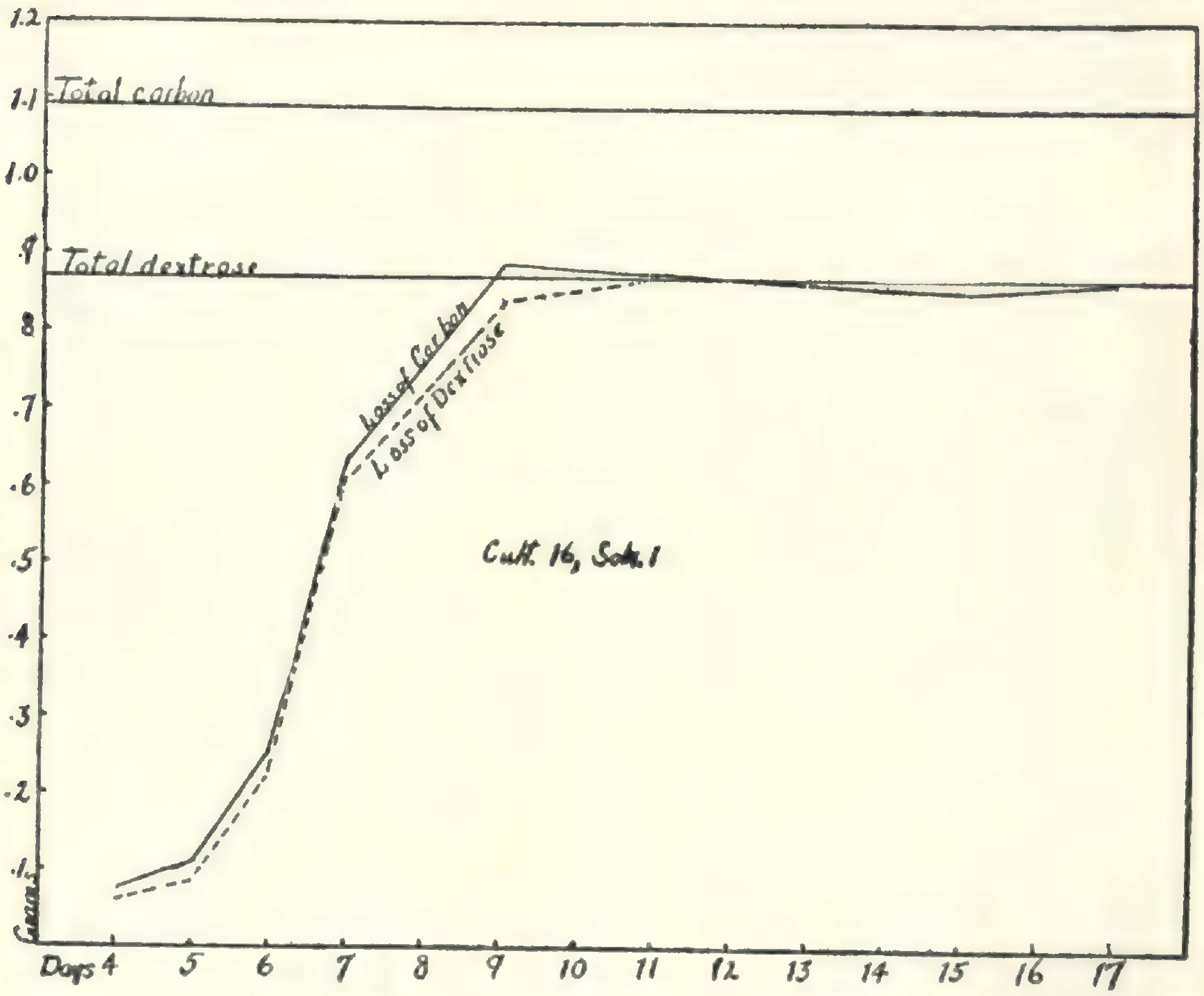


Fig. 10. *Penicillium digitatum* in solution 1.

It is impossible to draw clean-cut conclusions from the curves in fig. 11. The fact that the loss of citric acid as determined by analysis was greater than the "loss of carbon—loss of dextrose" would indicate that some product was being formed in the solution from either the citric acid or the dextrose, and, in all probability, from the former. That this substance could not be oxalic acid is obvious from the fact that by the method used oxalic acid would have been included with the citric acid in the barium precipitation. It was not a volatile substance, since it was oxidized in the carbon apparatus and, if an acid at all, it had a

soluble barium salt and very weak acidity, for the titration figures fell to 1.1 cc. of N/10 NaOH per 25 cc. of solution, and the P_H to 7.2. These facts preclude most of the common acids and give the impression that it was some other inert substance.

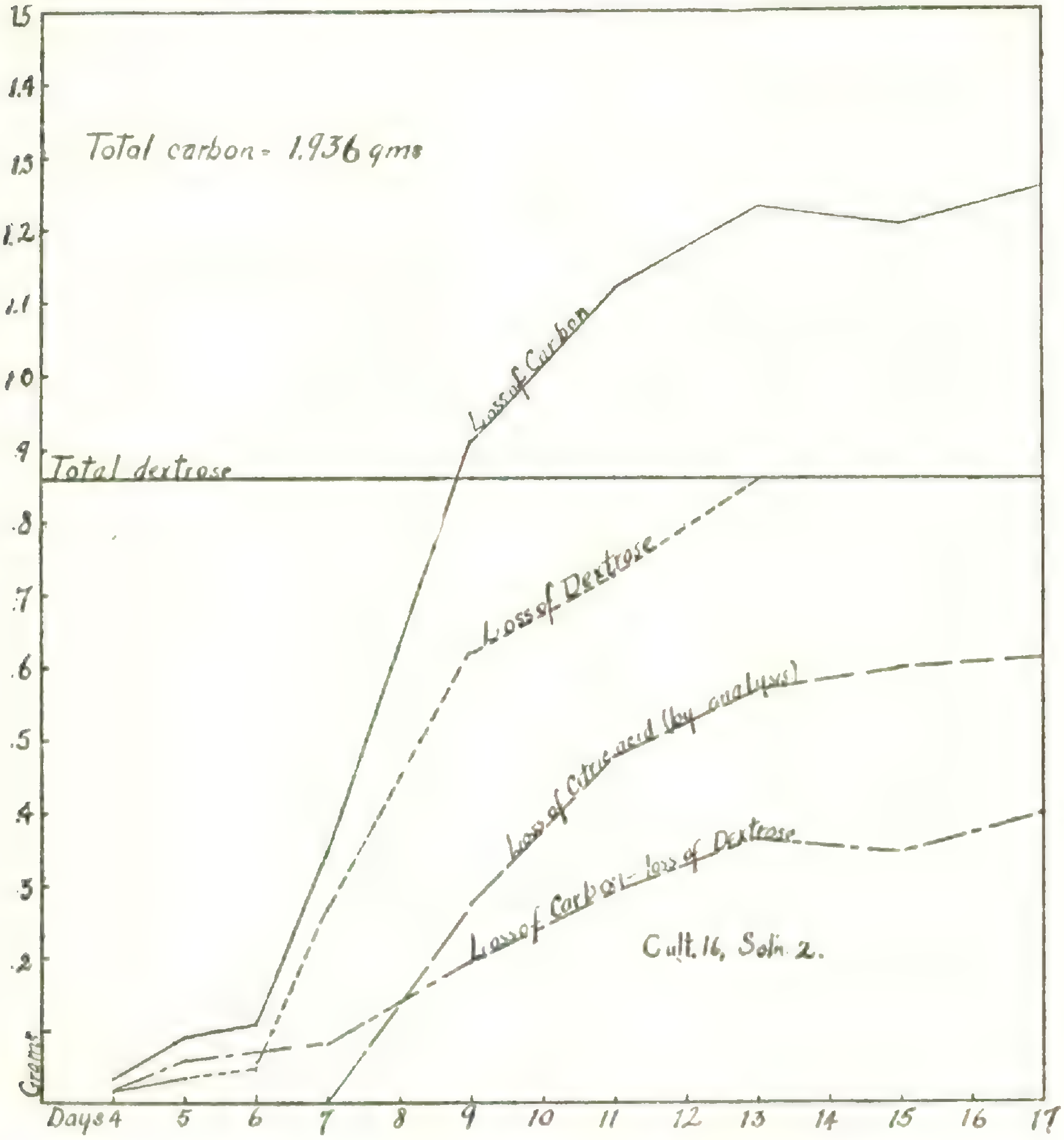


Fig. 11. *Penicillium digitatum* in solution 2.

Diplodia natalensis.—In figs. 12 and 13 are found the curves for the analytical work and in fig. 8 the curves indicating the trend of P_H during the period of growth. The solution used for this fungus was the same as that used for organism 2 except that NH_4NO_3 was substituted for KNO_3.

There was considerably greater growth in solution 2 than in

solution 1, almost one-half more, but it is noticeable that the peak of growth occurred after all the dextrose had disappeared, in solution 2, but was coincident with its disappearance in solution 1, or approximately so. Likewise, the weight of the mats fell off much more rapidly in solution 1 than in solution 2. Here again there was a greater weight of mat in solution 2 when the dextrose disappeared than in solution 1 at the same relative period.

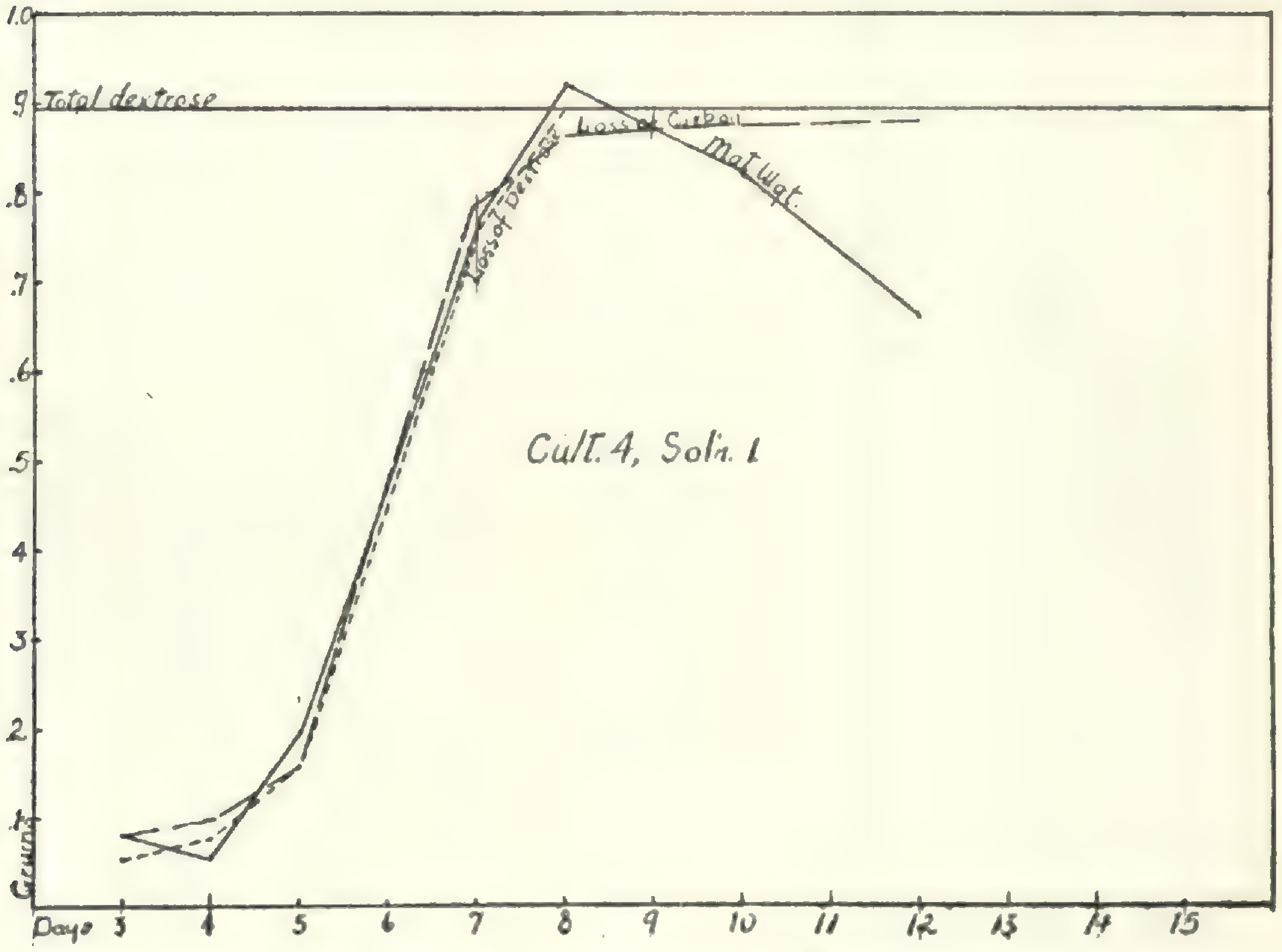


Fig. 12. *Diplodia natalensis* in solution 1.

The disappearance of dextrose came at about the same relative period, in both solutions. The data for solution 1 would indicate that little, if any, non-volatile compounds were formed. The "loss of carbon—loss of dextrose" curve for solution 2 proceeds below the base line for the first 8 days, indicating that a small amount of waste products was probably formed from the dextrose. Beginning on the ninth day this curve begins to rise rapidly, and from this point runs practically parallel to the curve calculated from the titration figures. Likewise, the point at which the curve for "loss of carbon—loss of dextrose" starts to ascend is coincident with the final disappearance of dextrose from the solution.

It was impossible to detect even traces of oxalic acid in this solution or of either citric or oxalic in the dextrose solution. However, in neutral solution, a gelatinous precipitate could be obtained

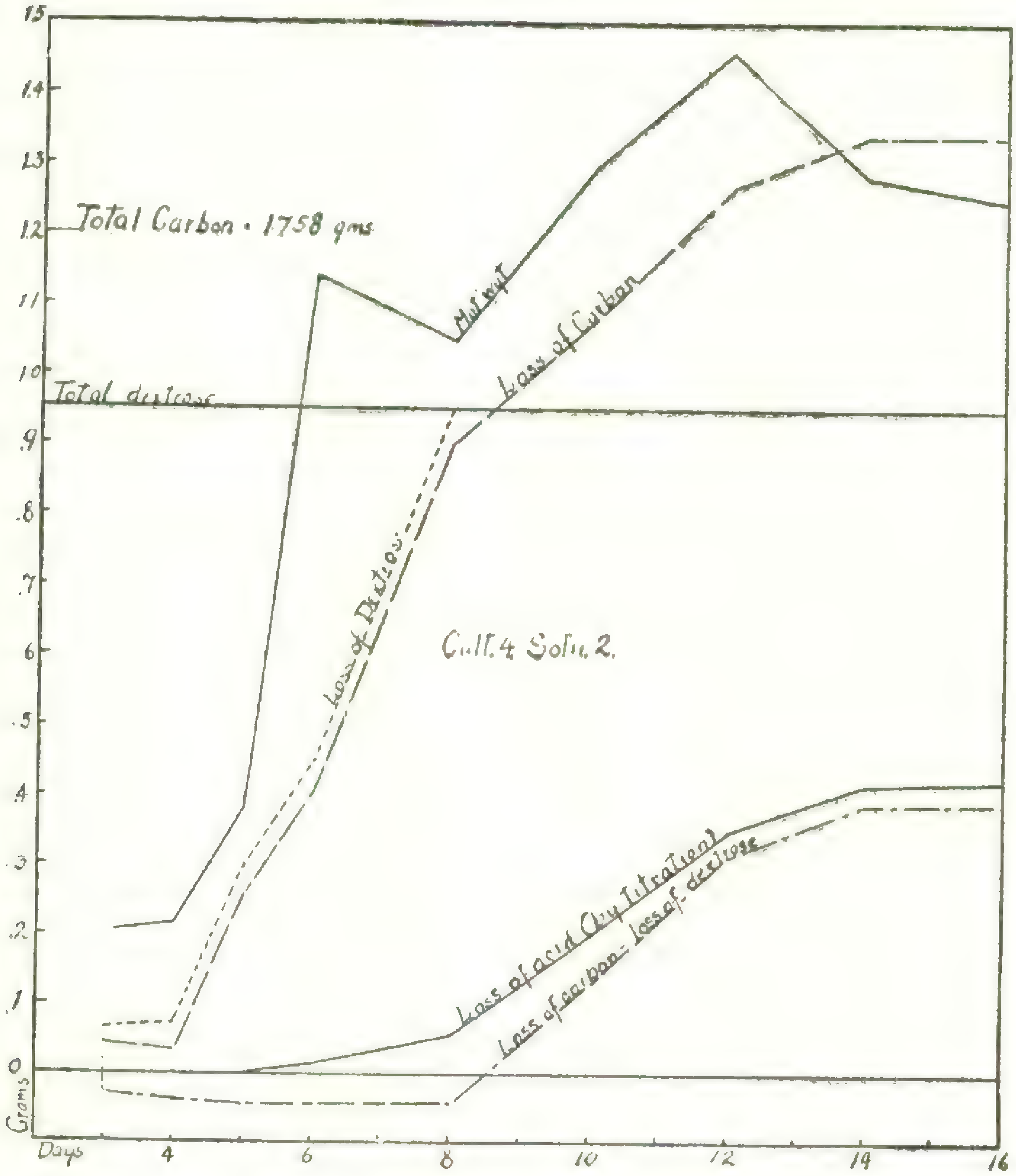


Fig. 13. *Diplodia natalensis* in solution 2.

with $Ca(OOC.CH_3)_2$. This precipitate had a wine-red color and dissolved with difficulty in dilute acids, did not reduce Fehling's, give a pentose reaction or show either alkaline or acid characteristics. Moreover, there seemed to be a considerable amount of gelatinous material in the solution in which the fungus was grow-

ing, making it very difficult to filter off the mat, the supernatant liquid going very slowly through coarse filter paper.

The curves for the trend of P_H are found in fig. 8. Solution 1 showed a marked rise of acidity followed by a falling off to alkalinity. Solution 2 did not rise in acidity but after 6 days began to fall off steadily toward alkalinity. Whether the development of acid was hindered by the buffer action of solution 2 or merely masked by it is a question that cannot be answered satisfactorily. It is to be seen that as the titer of the solution decreased to a very small amount, utilization of carbon ceased. This might have been due to the fact that the solution was too alkaline for the metabolism of this fungus or that the fungus could not utilize the citrate radical when combined with potassium.

Alternaria Citri and *Alternaria* sp.—These 2 fungi will be discussed together for the sake of comparison. The solutions contained KNO_3 as a source of nitrogen and 100-cc. flasks were used. In diluting, 2 flasks were made up to 200 cc. The P_H of the solution was about 4.7.

It is difficult to interpret the weight curves for these organisms, as seen in fig. 14. Organism 14 showed more rapid growth than did organism 7, and if the final high figure for solution 1 is taken into account would seem to have made a greater total growth on dextrose alone. However, as will be noted, the dextrose had entirely disappeared 16 days before this final high weight was obtained. There are only 2 reasonable explanations of this fact: the first, that this weight is accounted for by the irregularity of growth, and that these 2 particular mats would have weighed as high or higher at an earlier period; the second, that the mats for some reason were insufficiently dried and consequently were somewhat heavier than they should have been. While this last-mentioned explanation seems improbable, since the mats were run in large groups and no other difficulties were encountered, yet it seems wise to disregard such an unusually high weight occurring at this point. With regard to both of these fungi and other fungi of a similar nature there is considerable irregularity in the weights of the mats, presumably due to the slowness of growth, and consequently the greater likelihood of being influenced by factors of the environment. It would have been highly

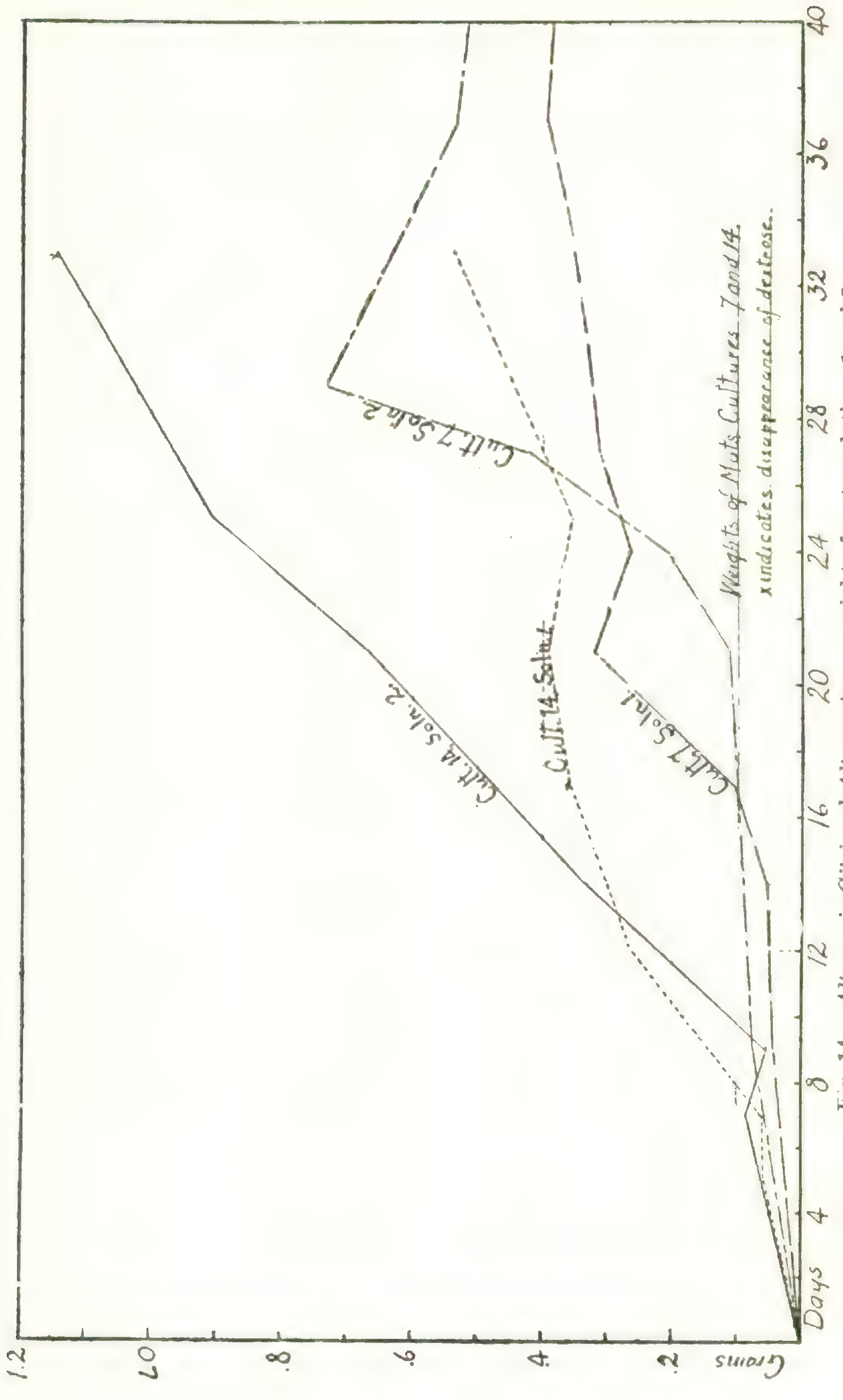


Fig. 14. *Alternaria Citri* and *Alternaria* sp., weight of mats, solutions 1 and 2.

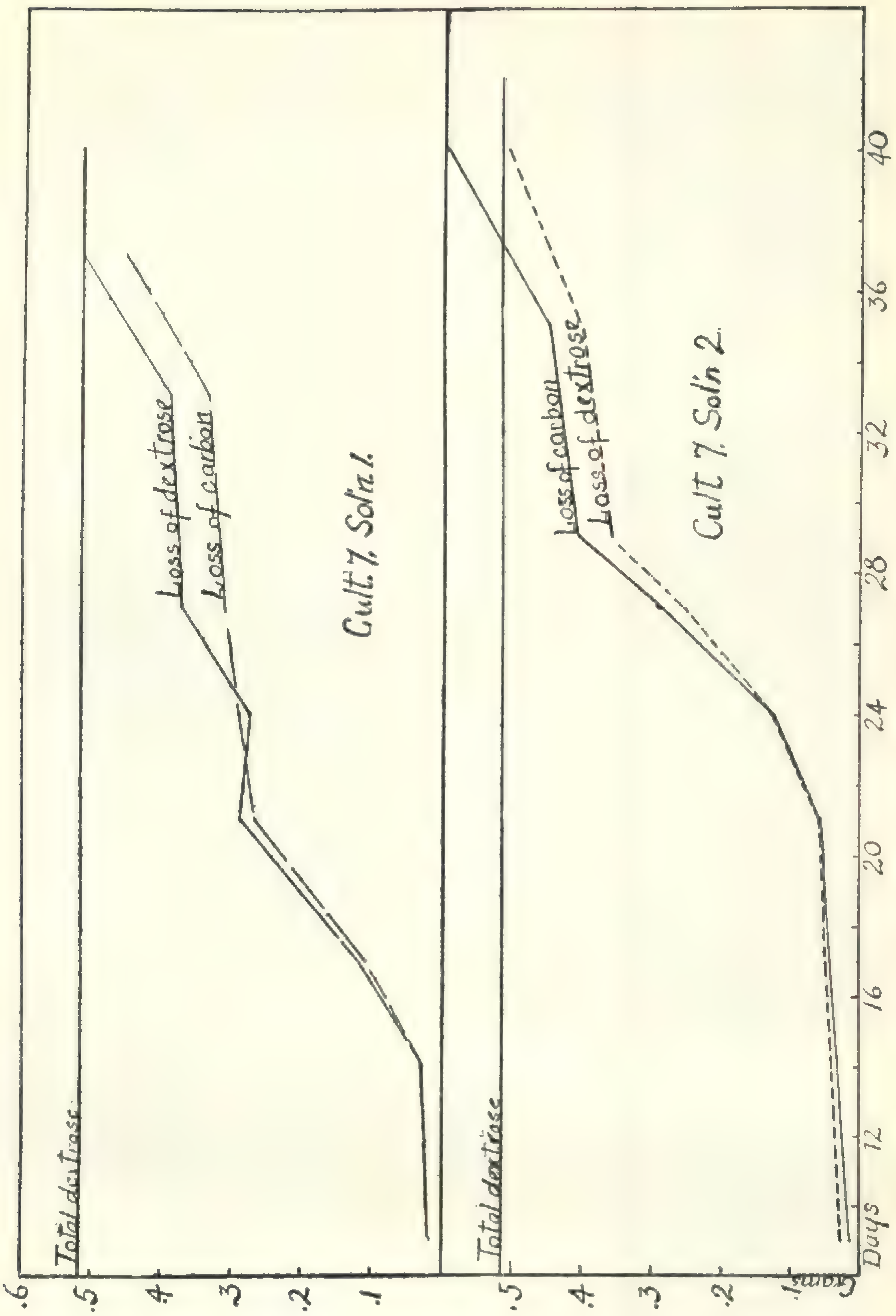


Fig. 15. *Alternaria Citri* in solution 1 (above) and solution 2 (below).

desirable to have had more cultures for each determination. Culture 14 gave much better growth in solution 2 than in solution 1, as well as a delayed utilization of dextrose in the former. This would indicate that the citric acid was used at the same time as the sugar and that the former had a high mat-building value.

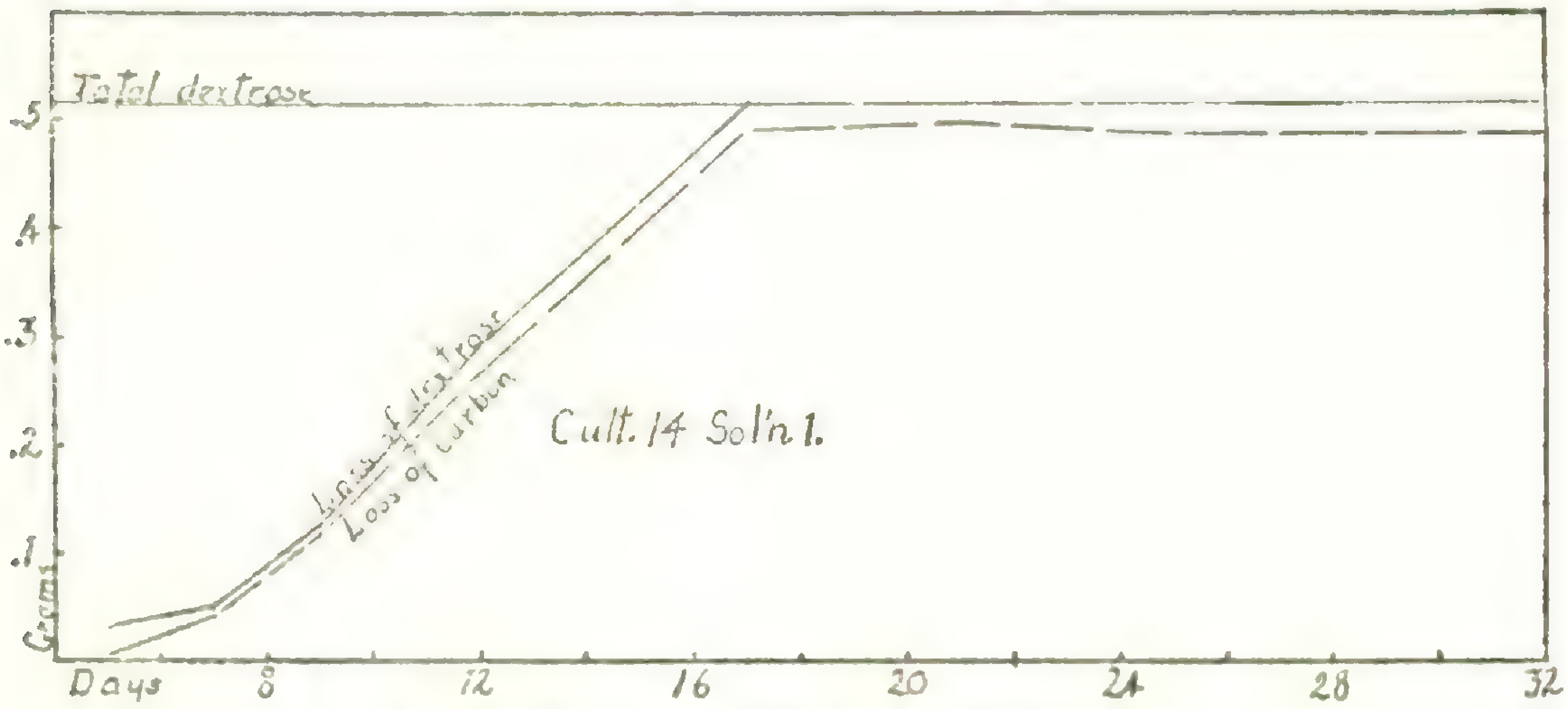


Fig. 16. *Alternaria* sp. in solution 1.

Unfortunately, contaminations prevented the completion of the curves. For organism 7 additional weights not shown on the curves were obtained as follows:

Day	Wgts. in mgms.	
	Solution 1	Solution 2
43rd		502
47th	341	486
50th	376	

These figures indicate that the maximum had already been reached, though there was still a slight trace of dextrose in solution 2 on the forty-seventh day. The maximum in solution 2 on the twenty-ninth day was apparently too great to represent an average culture, the peak of the curve being too sharp at this point. Where growth is as slow as it is with this organism, it is likely that there is reached a condition in which autolysis balances or even exceeds the amount of growth due to the utilization of the carbohydrate still remaining in the solution. It

seems reasonable to suppose that the smaller the amount of carbohydrate in the solution, in proportion to the absorbing surface, the more difficult it becomes for the fungus to absorb it in sufficient quantities to show a continued increase in growth. Where growth is very rapid, as in some species of *Penicillium*, this effect would be masked by the rapid and complete disappearance of the sugar from the solution, but in the case of *A. Citri* this difficulty would manifest itself to a greater degree.

The curves representing the daily change in P_H of the two solutions of organism 14 (figs. 16, 17) are very interesting and prob-

Fig. 17. *Alternaria* sp. in solution 2.

ably represent the typical results for this type of fungus. The curve for solution 1 rises very slightly and then falls off rapidly to a comparatively high alkalinity and remains there for the remainder of the duration of the experiment. In solution 2 the P_H remained constant for a considerable time and then showed a very slow falling off toward alkalinity. This maintaining of the P_H in the early stages of metabolism is an expression of the buffer activity of the citrate mixture; the rapidity with which the subsequent falling off occurs depends entirely upon the rapidity with which the citric acid is used. If in this case the buffer was not consumed at all by the fungus the stability would be even greater and an organism would be required to produce a considerable amount of either alkaline or acid substances to move

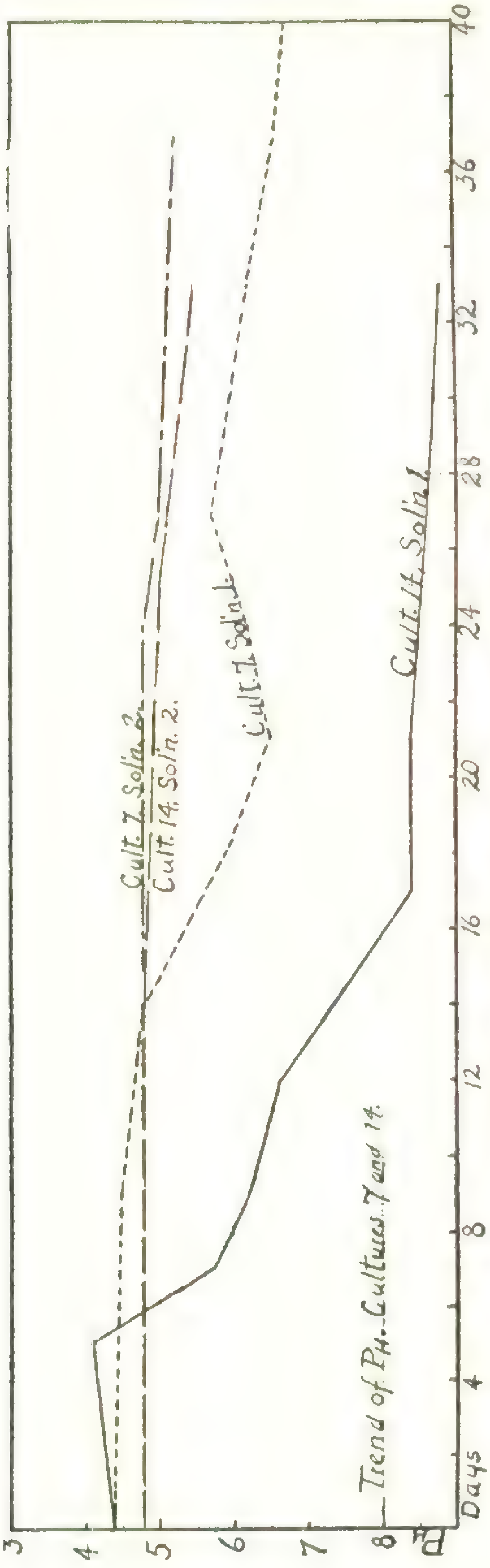


Fig. 18. Trend of P_H, *Alternaria Citri* and *Alternaria* sp.

the P_H through any considerable range of hydrogen-ion concentration. In solution 2, culture 7, we find the same buffering, but there is a marked difference between the curve for solution 1 and that for organism 14, solution 1. There was no rise of acidity at the start but a gradual falling off followed by a rise to a slight peak and a subsequent falling off. The rise in acidity at this late point might be considered only a variation in certain particular cultures were it not for the fact that several determinations were involved in this "peak."

For organism 14, solution 2, there was a steady rise in the "loss of carbon—loss of dextrose" curve, almost from the start. This rise is very slow, however, and the increased growth in solution 2 seems to be out of proportion to the amount of carbon used to produce it. How much the buffering rather than the use of the carbon from the citrate may account for this increase is a question that cannot safely be discussed until more data are available.

Generally speaking, organism 14 is the more active and rapid grower when compared with organism 7; likewise it makes more effective use of the citrate radical. Another marked difference between the two organisms lies in the curve of P_H in a medium in which dextrose is supplied alone, as noted above. Whether these fungi represent two species is a problem for the mycologist to decide however.

Sclerotinia Libertiana.—The growth curves for organism 11 are found in fig. 19 and the curves for the analyses in figs. 20–21. Owing to the slow growth of this fungus and the succeeding one (*Phomopsis Citri*) the culture solution was varied somewhat. Peptone was used as a source of carbon and solution 2 contained M/20 dextrose instead of M/4. Flasks of 100 cc. capacity were used instead of the 300-cc. flasks.

In the growth curve for solution 1 a sharp peak appears on the ninth day and coincident with it is found a peak in the "loss of carbon" curve and a complete loss of dextrose. On the ninth day it was assumed that there was no dextrose in the solution but the curve for "loss of carbon" indicates that there was probably considerable sugar present and that the peak on the ninth day indicated erratic cultures. In the normal culture the dextrose would probably be found to disappear about the fourteenth to

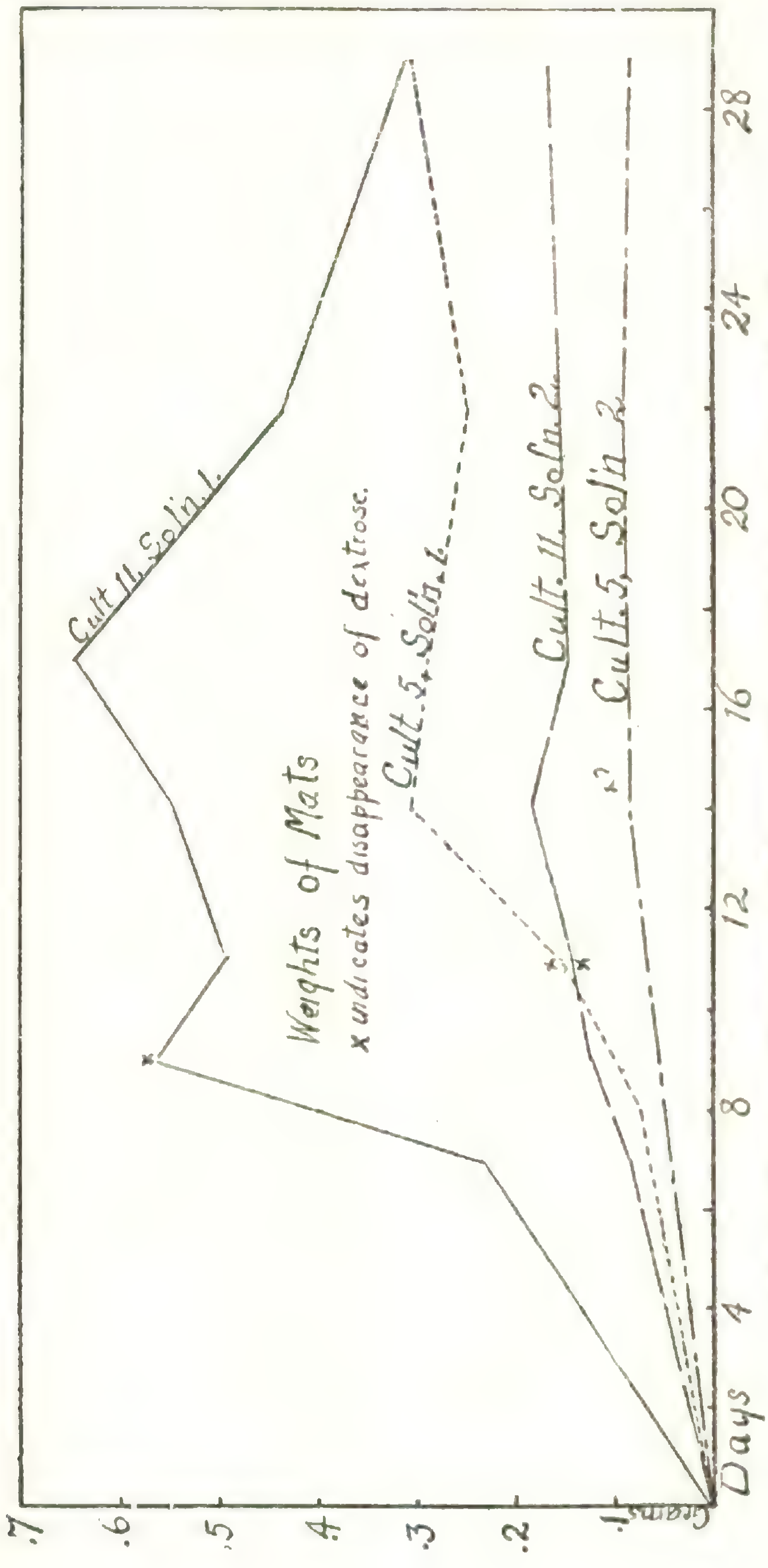


Fig. 19. *Sclerotinia Libertiana* and *Phomopsis Citri*, weight of mats, solutions 1 and 2.

sixteenth day under the same conditions. If no citrate were used the growth curve for solution 2, at its peak, would be expected to be about one-fifth the height of that for solution 1. As a matter of fact it is a little better than that and the peak is maintained instead of falling off as it did in solution 1. The curves

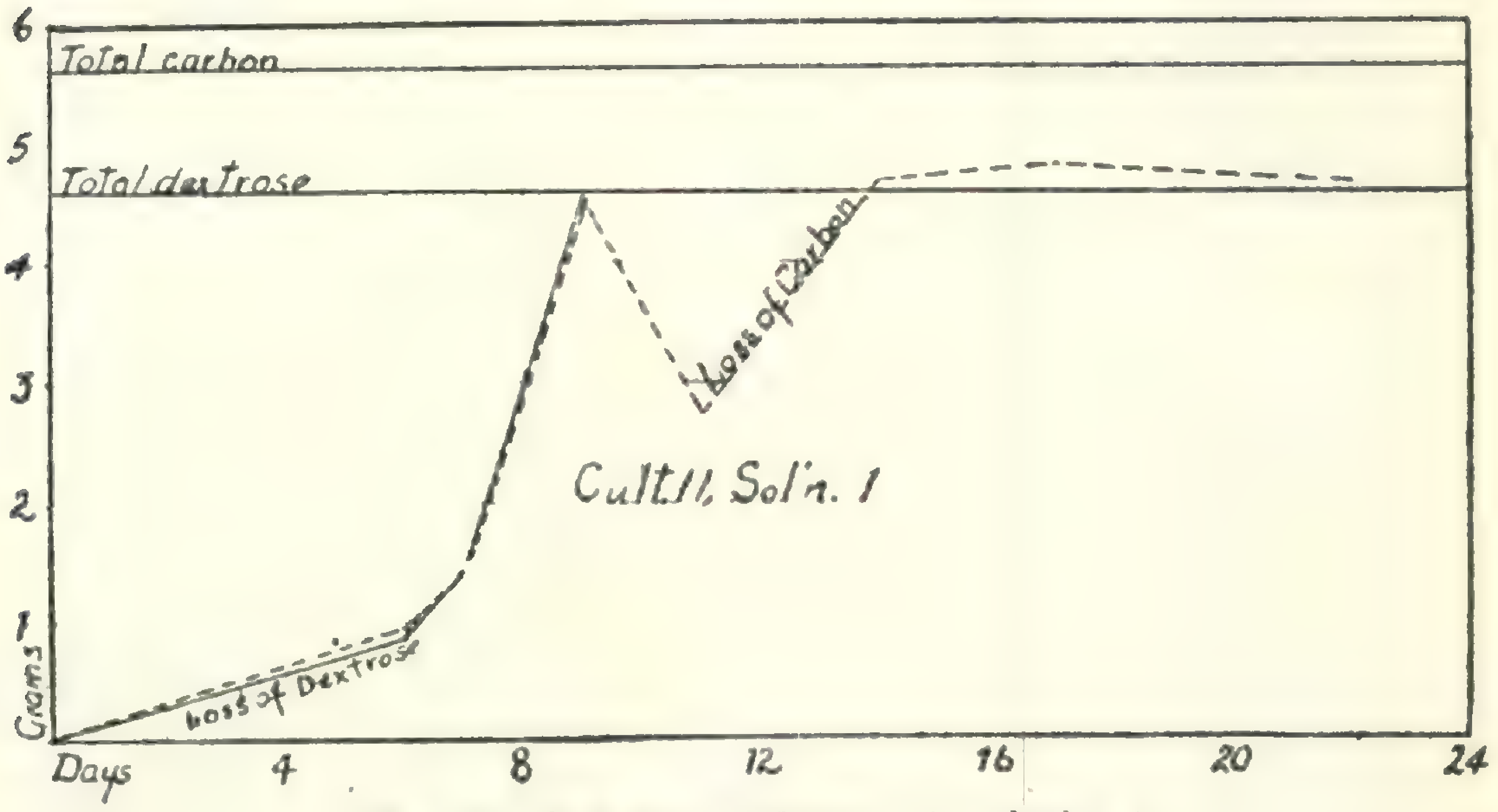


Fig. 20. *Seclrotinia Libertiana* in solution 1.

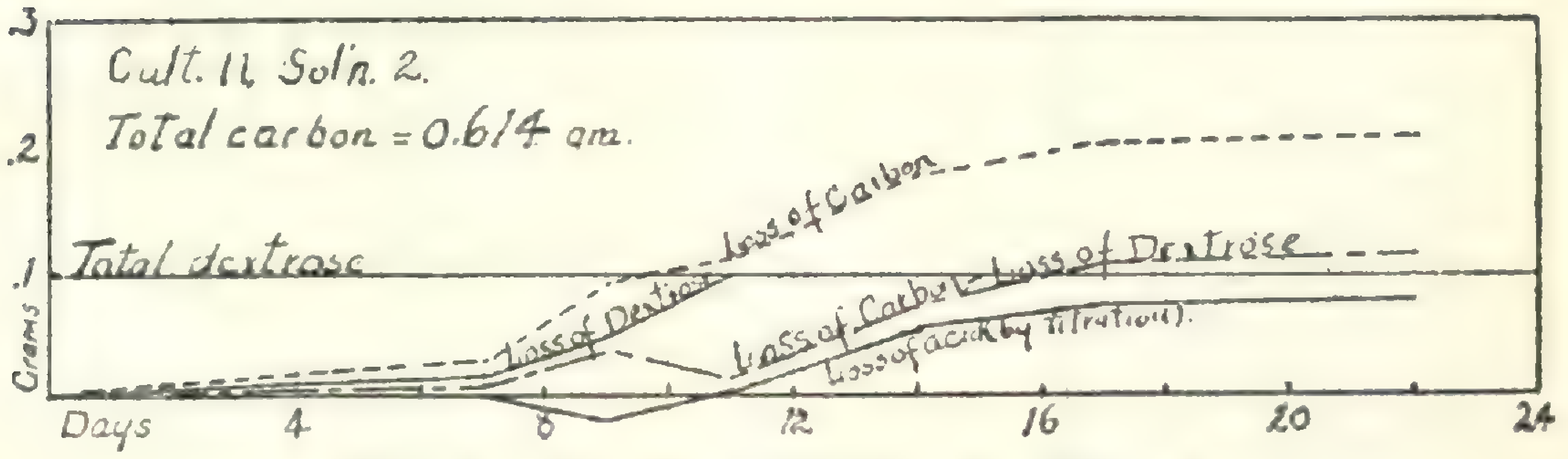


Fig. 21. *Sclerotinia Libertiana* in solution 2.

for the analyses indicate that a little citrate was used though not a great deal.

In the analytical curves for solution 1 (fig. 20) the "loss of dextrose" and "loss of carbon" curves follow each other closely, and in solution 2 the "loss of carbon—loss of dextrose" curve rises more rapidly than does the curve calculated from the titrations. The conclusion from the above circumstances is that the salt (*in toto*), as well as the free acid, was being used.

Phomopsis Citri.—The growth curves for organism 5 are given in fig. 19. The analytical curves are not given, as they held noth-

ing of particular interest, since, at the end of 22 days, no more than a trace of citrate had been used. The maximum weight of mat for solution 2 was a little more than a fifth of the maximum in solution 1. On the fourteenth day a good test for oxalic acid was obtained in solution 1.

The production of alcoholic products by various fungi.—In connection with the latter part of the work it was intended to carry out aeration experiments in which the fungi were to be grown in flasks plugged with rubber stoppers provided with 2 aeration tubes (inlet and outlet) and by this method to determine the amount of CO_2 produced. It was believed that by aerating the cultures twice each 24 hours the CO_2 tension would be kept sufficiently low to allow of normal development of the fungus and that enough O_2 would be furnished. However, organism 9, which was tried first, failed to develop rapidly and for a time refused to sporulate, and later sporulated only sparingly in contrast to the ordinary cotton-plugged cultures which sporulated heavily. As it seemed useless to continue the aeration experiment, due to the abnormal growth resulting from these conditions, the flasks were left stoppered for about a week without aeration. As the culture solution had developed a strong fermentative odor, it was poured off and distilled, in the hope of finding the cause of the odor. Twenty-five cc. of solution of markedly alcoholic odor were obtained by distillation from 150 cc. of culture solution; and on dilution to 100 cc. the solution had a specific gravity slightly lower than that of water and gave a benzoate with a very penetrating odor (Baumann and Schotten reaction). This odor somewhat resembled that of ethyl benzoate but seemed more penetrating. Weak iodoform tests were obtained in the cold and stronger on warming, following the instructions of Mulliken ('04). The crystals were examined microscopically and found to correspond with those given by ethyl alcohol by the same test but this is not distinctive. No similar products were obtained where only dextrose was present as the source of carbon. Lack of time and equipment for organic analysis prevented further study of this solution in an analytical way, but similar phenomena were observed in numerous cultures and it may be worth while to indicate some of the results obtained.

In cultures of *Phomopsis* in the presence of large amounts of citrates a marked yeasty smell suggesting ethyl alcohol and dilute acetic acid developed after a prolonged growth period; with only dextrose and peptone no such odor developed. Both of the *Alternaria* spp. in dextrose-citrate mixtures developed similar odors, and distillation gave solutions with the odor of alcohol. These distillates gave iodoform tests on warming and benzoates with the odor of ethyl benzoate, or suggesting mixtures of ethyl benzoate with allied benzoates. When a series in which KNO_3 was used as the source of nitrogen was inoculated with *Oospora Citri-aurantii* the growth was so slow and the difficulties connected with filtering off the organism so great that the series had to be abandoned. After about 2 weeks a very marked sweetish odor developed, nearly resembling slightly fermented cider, and this applied in less degree to the culture solution where only dextrose was used as the source of carbon. On distillation the odorous compound was carried over to the distillate and suggested an ester of ethyl or some closely allied alcohol.

These results are necessarily incomplete, yet merit some discussion. The factors which all these cases had in common were (1) a growing condition in which there was a tendency to anaerobic environment, and (2) citric acid-potassium citrate mixtures (with one exception—see *Oospora Citri-aurantii*). This semi-anaerobic condition was obtained in the *Aspergillus* cultures through stoppering the flasks with rubber stoppers and in the other cultures by the mat tending to form (where there was a mat formed) *in* the solution instead of *on* its surface as was usual with most of the fungi used. *Oospora* grows in the solution in a yeast-like manner; the two species of *Alternaria* and the *Phomopsis*, besides being slow growers, start under the surface of the solution and eventually form a sponge-like mass which contains the solution interstitially. Under such circumstances there is certain to be a solution saturated with CO_2 and at the same time a probable shortage of O_2 due to the slow diffusion from the air into the solution. A moderated condition of anaerobism in which there is an excess of CO_2 and a shortage of O_2 would seem then to be a primary factor.

The question of carbon source is more complicated than is

indicated at first glance. There are 2 solutions under consideration; one in which only dextrose was used as a source of carbohydrate, the other with dextrose and the addition of a comparatively large amount of citric acid-potassium citrate mixture. The alcoholic products occurred only where the citrate mixture was present, except in the case of *Oospora*, and the natural inference is that the citrate radical is the source of the products formed. However, it is within the field of possibility that this may not be the case, but that the strong buffer action of the citrate mixture is successful in maintaining the reaction at an unfavorable P_H for a considerable time, which might result in an abnormal metabolism of dextrose. However, in the *Phomopsis* cultures only a small amount of dextrose was present with the citrate mixture and this was soon utilized, probably before the solution had acquired the semi-anaerobic condition. Likewise, when the cultures of *Aspergillus*, which had been stoppered for the aeration work and which contained only dextrose and produced no appreciable amount of alcoholic products, had citric acid added to them after the dextrose was exhausted the odor of alcohol rapidly developed.

The question of buffering was raised primarily in connection with *Diplodia natalensis*. A series of flasks of the dextrose-citrate solution was prepared but made too acid for the good growth of this fungus. After inoculation the fungus developed very slowly tufts of floating mycelium in the solution and after about 2 weeks a small tuft on the surface. The odor of ethyl alcohol was very ttrong, and there was also present some other substance of penelrating odor but probably not acetic acid; on distillation the distilsate gave an immediate and very strong iodoform test in the cold, indicating that either acetone or isopropyl alcohol was probably present. As no benzoyl chloride was available at the time no benzoate was made. The odor of the distillate, however, suggested a mixture of ethyl alcohol and acetone together with some other substance. A similar solution adjusted to a more favorable P_H did not produce these odorous compounds. The inference here is that the buffering of the solution at an unfavorable P_H might be the important factor, but it is reasonable to suppose that the buffering should affect the metabolism of the citrate

radical as well as of the dextrose. Moreover, the results might be indirect in that the buffering at an unfavorable P_H caused the mat to form *in* instead of *on* the solution and that this was the direct cause of the incomplete metabolism.

Other cultures used failed to produce such evident volatile metabolic products, but both *Penicillium stoloniferum* and *P.* sp. produced some odorous compound which was given off when the solution was neutralized. This distilled over readily and formed a benzoate by the Baumann and Schotten reaction which had an odor of some rubber compounds.

These observations are very suggestive of a fruitful field for investigation in the future. Moreover, they merely add to the data indicating that the metabolism of fungi is far from a simple problem and that the end products may be in many ways independent of the conditions of the environment. It is quite probable that ethyl and isopropyl alcohols, acetic acid, and possibly acetone may be end products of the metabolism of citric acid by fungi and that they may also be the end products of the metabolism of sugars, providing the correct environmental conditions are provided. It is likewise probable that these environmental conditions have to do with the $O-CO_2$ relations and that any factors affecting this relation may have effect upon the ultimate products obtained.

Summary

An improved method for the determination of citric acid, especially applicable to culture solutions, has been offered.

The application of the wet combustion method, for the determination of total carbon, to physiological work has been indicated.

A number of fungi which attack citrus fruits have been studied with regard to their ability to utilize citric acid as a source of carbon with the following general results:

(1) None of the fungi tried was found to thrive on citrate as the sole source of carbon.

(2) Citrate mixtures adjusted to a favorable P_H proved to be efficient supplementary carbon sources, when used with small quantities of dextrose, for all the fungi used with the exception of *Penicillium digitatum* and *Phomopsis Citri*.

(3) After a mat had been grown on dextrose, free citric acid was utilized readily by *Penicillium stoloniferum*, *P.* sp., and *Aspergillus* sp., and somewhat less readily by *Sclerotinia Libertiana.* Free citric acid was used slightly or not at all by mats of *Diplodia natalensis*, *Phomopsis Citri*, *Alternaria Citri*, *Alternaria* sp. and *Penicillium digitatum.* The response of the fungi in this respect was coordinate with their tolerance of the hydrogen-ion concentration.

Studies have been carried out with *Penicillium* sp., *Penicillium stoloniferum*, *Penicillium digitatum*, *Aspergillus* sp., *Diplodia natalensis*, *Alternaria Citri*, *Alternaria* sp., *Phomopsis Citri*, and *Sclerotinia Libertiana* on a dextrose and a dextrose-citrate medium, and curves for the growth, trend of P_H, "loss of dextrose," "loss of carbon," etc., are given for these fungi.

Acidic and alcoholic products were found to be formed under unfavorable environmental conditions, that is, lack of O_2, unfavorable P_H, etc.

It has been pointed out that tolerance or utilization of free citric acid is probably not an important factor in the specialized parasitism of such fungi as *Phomopsis Citri*, *Penicillium digitatum*, *Alternaria Citri*, *Alternaria* sp., *Sclerotinia Libertiana*, and *Diplodia natalensis*, but that it probably is a factor in the destructive rotting of injured fruit by numerous fungi which cause this final collapse, and that under suitable environmental conditions *Penicillium stoloniferum*, *Penicillium* sp., and *Aspergillus* sp. would probably come in the latter category.

It is with great pleasure that the writer takes this opportunity for thanking Dr. B. M. Duggar for invaluable aid and advice in the prosecution of the work here reported; he also wishes to thank Dr. P. A. Shaffer of the Medical School of Washington University for aid in connection with the chemical work, and Dr. George T. Moore for the privileges and facilities of the Missouri Botanical Garden.

Graduate Laboratory, Missouri Botanical Garden.

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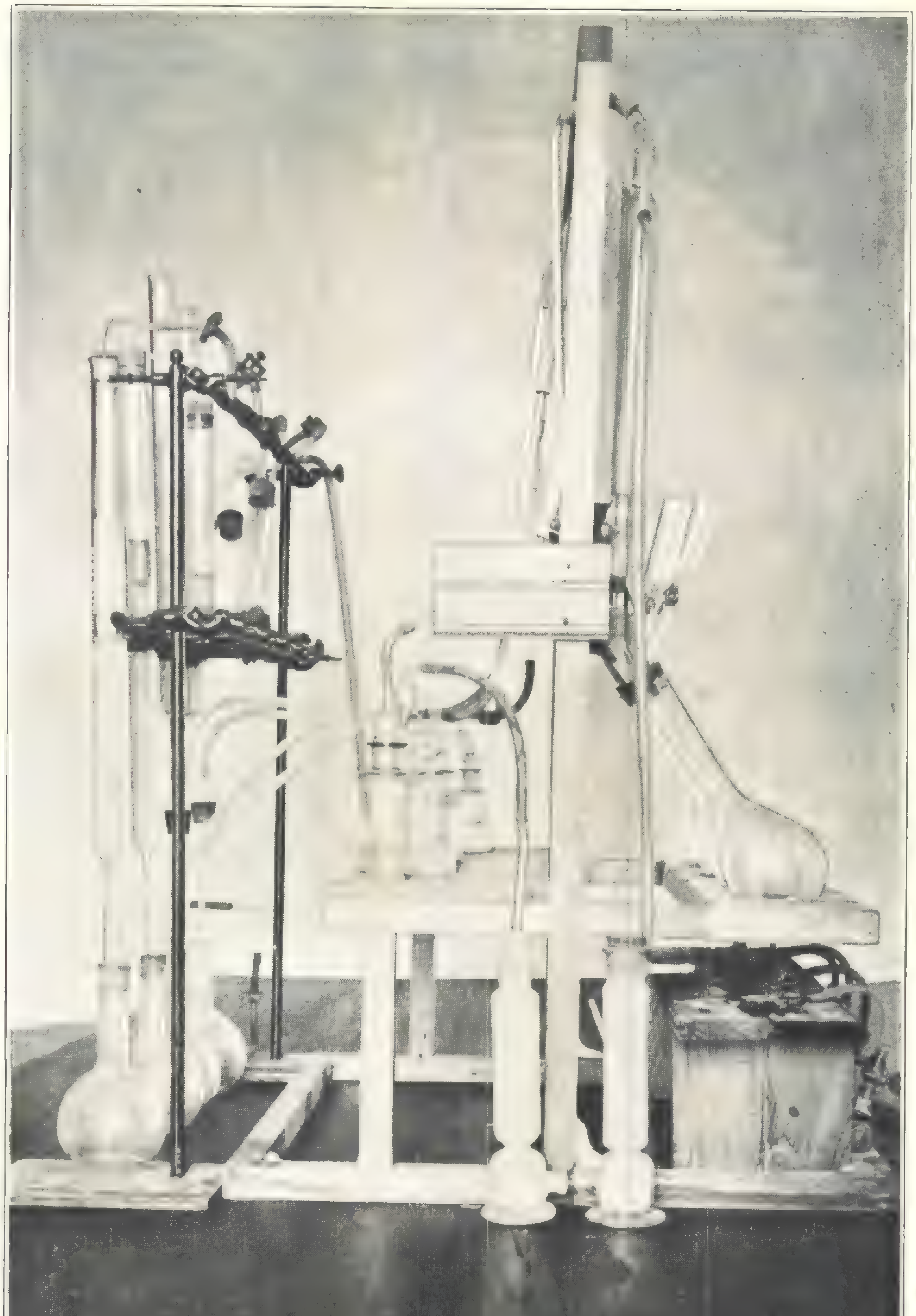
Explanation of Plate

PLATE 14

Carbon analysis equipment (see page 237).



CAMP—CITRIC ACID AS A SOURCE OF CARBON

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STUDIES IN THE PHYSIOLOGY OF THE FUNGI

XVI. Some Aspects of Nitrogen Metabolism in Fungi[1]

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Introduction and Review of Literature

Few extensive, really fundamental contributions to our knowledge of the nitrogen metabolism of plants are to be found. Even to-day practically nothing is known of the course of the assimilatory processes beyond the beginning and end points. It is evident that an insight into this fundamental life process will be more readily obtained by a study of fungi rather than of the higher plants, because of the relatively greater ease of the application of pure culture methods to the former.

The nitrogen fixation of fungi may properly be considered first. A glance at the literature reveals many conflicting data. In spite of the great number of publications, covering a period of over 50 years, it was not until 1916 that a paper appeared which included a complete review of the literature and gave evidence of adequacy in the technique employed. The technique of Duggar and Davis ('16) was significant in that Kjeldahl flasks were used directly as the culture vessels, thus necessitating no transference of either the fungous mat or culture solution prior

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy in the Henry Shaw School of Botany of Washington University.

to the nitrogen determination, a precaution which was not observed in the work of previous investigators. As many determinations were made on control portions of the media as on cultures of the various fungi. Under the several conditions of the experiments the fixation of elementary N by *Aspergillus niger*, *Macrosporium commune*, *Penicillium digitatum*, *P. expansum*, and *Glomerella Gossypii* could not be demonstrated; whereas *Phoma Betae*, growing on mangel and sugar beet decoctions with sucrose, showed a definite fixation ranging approximately from 3 to 8 mgm. per culture. This latter fact lends weight to the data of Ternetz ('04) indicating the marked capacity of *Phoma radicis* for assimilating free nitrogen. It, moreover, "throws open the whole question for any and all fungi," and suggests the sphaeropsidaceous and mycorhizal forms as material for first investigation.

The other important contributions to the subject of nitrogen metabolism are here reviewed, the chronological order being observed in so far as it was found compatible with clearness. Critical evaluation of the cited articles is largely reserved for the discussion at the end of this paper. The work with bacteria is not considered. The recent developments of physiological technique, such as the determination of active acidity and the improved methods for determining sugars and the various forms of nitrogen, throw doubt on the validity of the interpretations of many of the results of earlier investigators whose criterion for the assimilability and nutritive value of a given compound was generally based solely on the amount of growth. One now avoids arbitrary cataloging of fungi as "ammonia organisms," "peptone organisms," "nitrate forms" and so on, as was done in the older texts on plant physiology.

That heterotrophic plants were early known to be capable of utilizing inorganic nitrogen is evident from the challenge of Pasteur to Liebig to grow any amount of ferment on a purely synthetic medium which had ammonium N as the sole N source. Duclaux ('64), speculating on the synthesis of proteins in the course of fermentation, said that it was highly improbable that it could follow by direct condensation of ammonia and sugar, but rather that there must precede a breaking up of the sugar

molecule into its very reactive fragments which would unite with the ammonia to form amino acids from which the proteins would arise.

Czapek ('02), in his extensive cultural studies with *Aspergillus niger,* used cane sugar as a carbon source and an almost all-inclusive list of possible N sources. Judging from the substances used, the per cent of N utilized, the appearance of the culture, and the dry weights of the fungous crops obtained in the presence and absence of sugar, he concluded that proteins are most easily synthesized from the amino acids and from those substances which most nearly resemble the amino acids. For example, the high utility of acetamid as an N source is explained by the fact that its structure approaches that of an amino acid.

"Die Eiweissynthese wird demnach aus Aminosäuren (und Diaminosäuren) am leichtesten und ausgiebigsten bewerkstelligt, wenn man gleichzeitig eine gute Kohlenstoffquelle, z. B. eine Zuckerart, darreicht. Es vermag also der Schimmelpilz aus irgend einer beliebigen Aminosäure viel leichter alle übrigen, welche als Bausteine des Eiweissmoleküls in Betracht kommen, zu bilden, als erst aus anderen Stickstoffverbindungen Aminosäuren synthetisch aufzubauen. Man kann ferner annehmen, da die Aminosäuresynthese als Vorstufe zur Eiweissbildung anzusehen ist, dass aus jenen Stoffen, welche am besten als N-Quellen dienen (hier besonders den Oxyfettsäuren), auch am leichtesten die Synthese der Aminosäuren bewerkstelligt werden kann—ein Gedanke, welcher in ferneren Untersuchungsreihe noch weiter verfolgt werden soll."

In a later experiment he used asparagin as the N source and varied the carbon source, employing in all 24 carbohydrates, higher alcohols, and organic acids. From the dry weights obtained, the extent of utilization of the asparagin, and the appearance of the culture, he supports the "Eiweissregeneration aus Asparagin und Kohlehydraten" hypothesis of Pfeffer ('72) and Borodin ('78), stating that protein synthesis is essentially the same in *Aspergillus niger* as in the seedlings of flowering plants. Loew also speculated on the significance of asparagin in nutrition, saying that a fungus fed this and sugar forms an aldehyde of aspartic acid which is then condensed to make the protein molecule.

The classic chemical work of Emil Fischer (K. Hoesch, '21), following closely upon that of Czapek, threw much light upon the constitution of the protein molecule and its manner of synthesis.

Lutz ('05), using a modified Raulin's solution as the basis of a culture medium, compared the assimilability by *Aspergillus niger* of NH_4 salts, amines, amides, and nitriles by obtaining dry weights of the felts for a given period of incubation. He concluded that amides were the most assimilable, exceeding the NH_4 salts; amines came next, and the order of their assimilability was in inverse ratio to the size of their molecule; while nitriles were of little value. He stated: "This conclusion is in perfect concordance with that which we know of the chemical constitution of these diverse bodies; those in which the molecule is the more simple theoretically ought to be and practically are the better source of N for the plants."

Ritter ('09) worked with 8 different fungi, and employed, as criteria, dry weight and the quantity of 0.1 *N* alkali required to neutralize 10 ml. of the culture fluid after growth of the organisms. He formulated the following conclusions: (1) The weaker and less poisonous the free acids the better NH_4 is taken up out of its mineral salts. (2) The development of fungi on NH_4-salt solutions is in direct proportion to their ability to withstand free acid. (3) In relation to the quantity of mineral acids they are capable of generating, the fungi are placed in 2 groups: (a) the mat-forming fungi, as *Aspergillus* and *Rhizopus*, which liberate more acid than would permit the germination of their spores, and (b) such fungi as various species of *Mucor*, which grow submerged and produce acid in concentration not inhibitive to spore germination. (4) *Aspergillus glaucus, Mucor racemosus,* and *Cladosporium herbarum*, designated "Nitratpilze," develop as well on ammonium (NH_4).N. (5) These 3 fungi, however, show a strongly evident capacity for NO_3 assimilation; *Aspergillus niger, Botrytis cinerea,* and *Penicillium* spp. are weaker in this capacity and produce greater growth on $(NH_4)_2SO_4$; a third group, represented by *Rhizopus nigricans, Mucor Mucedo,* and *Thamnidium elegans* refuse nitrates.

In the 1912 paper he continues his ('09) observations, here employing other carbon sources than the grape sugar of the former work. By an ingenious method of draining the culture fluid off the mat and flooding the fungus with an alkaline nitrate solution, then incubating 2 or 3 days, he demonstrated the significant re-

duction of nitrates to nitrites by the various fungi. The solution was tested qualitatively with Trommsdorf's reagent and metaphenylendiamin. Nitrite was considered an intermediate product in nitrate assimilation. Ammonia, however, evolving from further reduction could not be demonstrated. Because ammonia is a product of autolysis he warns against the error of interpreting the presence of this as an indication of further reduction, as was done by Schlösing and Müntz ('78), Hagem ('10), and others.

Ritter ('14) grew *Aspergillus niger* on a medium containing 10 per cent cane sugar as the carbon source, and NH_4NO_3 in concentrations of 0.4, 0.8, 1.6 per cent as the source of N. After incubation the mat was filtered off, washed, and dried, and on the filtrate and washings, made to volume, were determined NH_3 by distillation with MgO, RNO_3 by reduction with Zn and Fe, and acidity in terms of 0.1 *N* NaOH. The acidity was also calculated from the difference between the amounts of RNO_3 and NH_3 found in the culture. He found that the extent of the acidity attained in 3 to 4 days was such as to render further development of the fungus impossible, as shown by the dry weights. The quantity of acid produced in the media containing 0.8 and 1.6 per cent NH_4NO_3 approached 0.1 normality but in those having only 0.42 per cent it reached only 0.04 *N* in 2 days and fell to 0.002 *N* in 8 days, indicating, he thought, a utilization of HNO_3 by the fungus. Parallel experiments with the N sources, ammonium tartrate, and HNO_3 in the strength equivalent to 11.35 mg. of N per culture, showed that the free acid after 6 days of incubation at 32° C. was superior for *Aspergillus niger* and a *Penicillium.*

Hagem ('10) investigated many species of *Mucor* and divided them into 2 classes with respect to their ability to assimilate N from nitrates and nitrites. All forms that were capable of assimilating nitrates could also obtain their N from the NO_2 ion. Because of this and because all thrived on N supplied as ammonium salts, and because in all cultures ammonia accumulated in the culture medium, he assumed that in the process of nitrate assimilation nitrates are reduced to nitrites and further to ammonia. Species thriving on sucrose could not utilize this sugar

when amino acids were supplied as the sole N source. Amino acids, moreover, as the sole source of both C and N had very little value for these forms.

Kossowicz ('12), in his cultural studies with 10 fungi, including a *Botrytis*, several species of *Penicillium*, a *Phytophthora*, and 2 species of *Aspergillus*, found that KNO_3 formed a good N source in the presence of cane sugar or dextrose. All these forms likewise made good growth with urea or uric acid as the N source. The following exceptional things were noted. With cane sugar as the C source *Cladosporium herbarum* would grow neither on glycine nor hippuric acid, and *Penicillium crustaceum*, *P. brevicaule*, and *Aspergillus glaucus* also failed to grow on the latter acid. All 4 of these fungi, however, grew on both acids in the presence of dextrose or mannite. For a number of the fungi glycine, uric and hippuric acids were found to serve to a small extent as sources of both C and N. Extracts of several of the fungi each fermented uric and hippuric acids, showing that the process is enzymatic. His 1914 contributions may be summed up as follows. Using the same 10 fungi, he found urea, uric acid, hippuric acid, glycine, guanine, guanidine compounds, nitrites, nitrates, and $CaCN_2$ serviceable as N sources in the presence of cane sugar, the $CaCN_2$, however, only weakly. Uric acid, hippuric acid, glycine, and guanine in the presence of a mixture of mineral N sources (KNO_3, NH_4NO_3, and NH_4Cl) served as C sources, but urea, guanidine, and KSCN failed to do so.

Growing the fungi on a KNO_3-sucrose medium and at intervals testing for nitrites, Kossowicz obtained positive indications in all cases but very irregularly. Ammonia formation was evident in cultures of *Aspergillus niger* and *A. glaucus*, *Cladosporium herbarum*, *Penicillium glaucum*, and *Fusarium* sp., but the fact that he did not obtain NH_3 in 21 days' incubation, as shown by his seventh experiment, indicates autolysis as the cause for its presence. Distillation with MgO was used for NH_3 determination, 20 ml. of the culture solution being taken; the Gręiscz and the Zn-iodide-starch methods were employed for the qualitative nitrite test and Nessler's reagent for NH_3. Of 7 different yeasts tried the formation of nitrite from nitrate could not be established for any. Nitrate was found a poor source of N for yeasts.

Brenner ('11) gave neither his biochemical methods nor any tables of results in his paper dealing with the nutritive value to *Aspergillus niger* of 30 various N sources. He stated that the assimilability was judged by determining the time required for the cultures to reach a maximum weight. Not considering the C of the organic N compounds, dextrose was the sole C source employed. The temperature of incubation was 35° C. All N sources were used in the concentration equivalent to 0.5 per cent NH_4Cl. Free NH_3, $NaNO_2$, ammonium valerianate, and KCN were poisonous to this fungus in the strength used. Tetramethyl-ammonium chloride, nitroguanidine, nitromethane, isoamylacetate, pyridine chloride, and piperidine chloride were not assimilable. Four groups were made in the descending order of their assimilability:

1. Ammonium lactate, ammonium tartrate, asparagin, ammonium succinate, and ammonium oxalate.

2. NH_4 salts of H_2SO_4, HCl, HNO_3, and H_3PO_4, likewise carbamide.

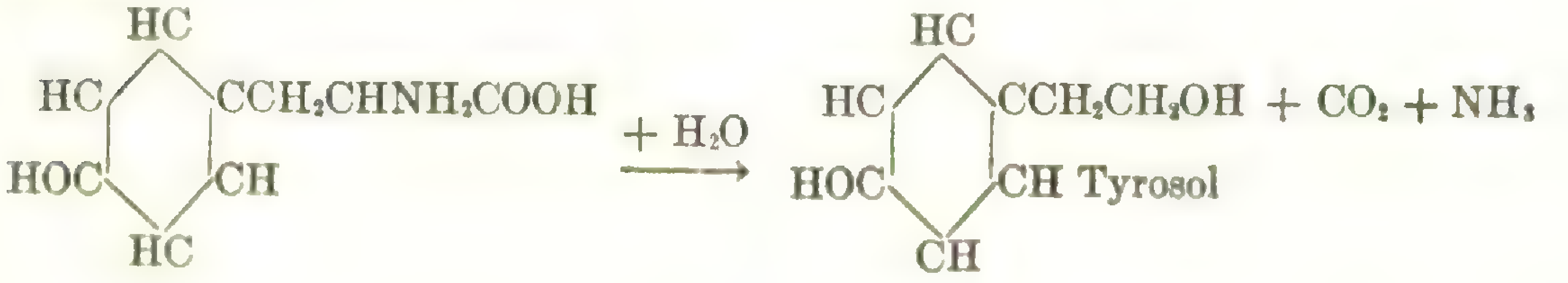
3. CH_3COONH_4, $HCOONH_4$, nitrosodimethylamine chloride, $NaNO_3$, pyridine nitrate, normal and isobutylamine chloride, guanidine nitrate, and chloride.

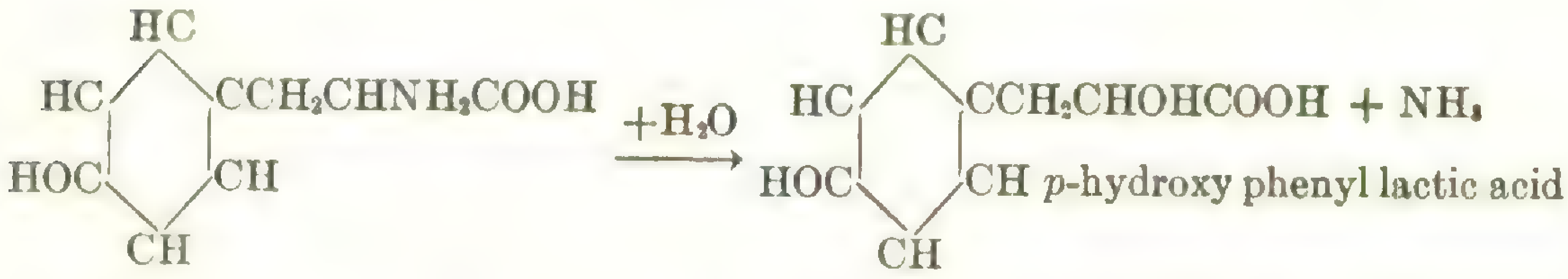
4. Isoamylamin chloride, hydroxylamine sulphate,benzylamine sulphate, dicyandiamid acetonitrile.

He stated that his study of the composition of the fungus and changes in the medium showed that after a growth period of about 4 days degenerative processes began in parts of the fungus. These were accompanied by the secretion of N as NH_3 or organic N. As a rule, regardless of the nature of the N source, about one-half of the N present in a solution containing the equivalent of a 0.5 per cent NH_4Cl solution was taken up by the first crop of the fungus grown. Subsequent crops, having less N at their disposal, contained a lower percentage of N than the first crops on the same solution.

A clearer conception of the course of protein synthesis in yeasts and other fungi is given by the illuminating qualitative and quantitative work of Ehrlich ('09, '11, '16, '17) and his associates. Following the lead of Duclaux ('64) and Pasteur ('58, '59) Ehrlich found that yeast could transform amino acids into alcohols

having one less C atom than the corresponding amino acids. Filamentous fungi, on the other hand, decomposed the amino acid to the corresponding hydroxy acid. Yeasts, especially *Willia anomala* and wild yeasts, were employed, as was *Oidium lactis*, *Rhizopus nigricans*, *Aspergillus*, and other fungi. Tyrosine, for example, was found to be hydrolytically decomposed by yeasts and fungi respectively, according to the following equations:





The technique by which the alcohol and hydroxy acid were isolated, identified, and quantitatively determined were given. The yield in some cases was almost quantitatively equivalent to the amount of amino acid consumed; that is, the N of the fungous mat determined by Kjeldahlization plus the non-nitrogenous complex, tyrosol, corresponded reasonably to the added amino acid, tyrosine. The ammonia formed is used up by the organism in its building of protoplasm and is not detectable in the culture fluid. The tyrosol produced in the presence of other carbon sources is not further used, but is really a by-product which diffuses out through the plasma membranes of the yeast cells.

Other amino acids were tried with similar results; for example, from tryptophane (β indolalanine) was obtained tryptophol (β indolethyl alcohol), a new compound whose properties were described in detail (Ehrlich, '12). Likewise from leucine and isoleucine yeasts formed amylalcohol and active amyl alcohol respectively. That the amino acids probably served solely as a source of N to the organism was indicated by the fact that upon the addition of ammonium salts to the medium the amino acids were not utilized. Boas and Leberle ('17), in this con-

nection, also found that *Aspergillus niger* utilizes only $(NH_4)_2SO_4$ as a source of N when both this and acetamide are present in the medium, and only glycine when this and acetamide form the N supply. They, moreover, found that $(NH_4)_2SO_4$ was used in preference to peptone in spite of the harmful effects of the acid freed in the utilization of the ammonia part of the $(NH_3)_2H_2SO_4$.

In Ehrlich's 1909 investigation yeasts were found to ferment glutamic acid to succinic acid; this was explained as an oxidation rather than an hydrolysis.

$$\begin{array}{l} CH_2COOH \\ | \\ CH_2 \\ | \\ CHNH_2COOH \end{array} \xrightarrow{+ O_2} \begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} + CO_2 + NH_3$$

Reactions that did not run as smoothly as these cited were explained by assuming that substances arising first were either unstable or suffered a change through secondary reactions.

The conclusion may be summed up in the words of the investigator:

"Nach den vorstehenden Ausführungen kann es keinem Zweifel unterliegen, dass auch bei der Assimilation von Aminosäuren durch Hefe der Aufbau ihres Plasmaeiweisses nicht anders erfolgt, also wenn der Hefezelle nur Ammoniak und Zucker dargeboten werden. Das die Hefe imstande ist, mit Ammoniak als alleiniger Stickstoffnahrung und Zucker also einzigem kohlenstoffhaltigem Material auszukommen und sich darauf normal fortzuentwickeln, haben ja bereits die Arbeiten Duclaux's einwanfrei erwiesen. Für den analogen Vorgang beim Wachstum auf Aminosäurenlösungen liess sich experimentell indirekt dadurch ein überzeugender Nachweis erbringen, dass es gelang, durch Zusatz von Ammonsalzen zu der gärenden Flüssigkeit auch im Überschuss vorhandene Aminosäure vor dem Angriff durch die Hefe zu schützen und dementsprechend die Menge der sonst auftretenden Abbauprodukte wie Fuselöl, Bernsteinsäure, Tyrosol u.s.w. auf ein Minimum zurückzudrangen. (See under "discussion," p. 350.)

Ehrlich and Pistschimuka ('12) extended this thought further to the utilization of primary amines by yeasts and fungi. Analogous to the fermentation of the amino acids, the primary amines underwent a similar decomposition to alcohols by hydrolysis of the amido group and splitting off of ammonia, which alone was used by the organisms.

$$RCH_2NH_2 + H_2O \longrightarrow RCH_2OH + NH_3$$

Tyrosol and fusel oil respectively were obtained from *p*-hydroxy-phenyl-ethylamine and isoamylamine. Guggenheim and Löeffler also showed this to take place in animal tissues.

Ehrlich ('16) then worked with the trimethylated amino acid, betaine, the secondary amine, adrenalin, and the tertiary amine, hordenin, and found the utilization of these compounds by fungi and yeasts analogous to the above. From betaine was obtained glycollic acid and trimethylamine, the latter being hydrolyzed by the organism to methyl alcohol and the directly usable ammonia. Adrenalin was hydrolyzed to *m*-*p*-dihydroxyl-phenyl-ethylene glycoll and monomethylamine; the poisonous hordenin, to the harmless tyrosol and dimethylamine, the amines in both cases, as with betaine, being further hydrolyzed to NH_3 and CH_3OH. In concluding, this investigator emphasized the use to which fungi may be put in the preparation of organic compounds otherwise difficult or impossible to prepare. He also pointed out the possibility that these reactions might throw light on the question whether the alkaloids of green plants, which are rich in alkylamines, are end products of metabolism or merely intermediate compounds which undergo a further change. He then emphasized the known relation of betaine and glycollic acid in sugar beets.

Completing the analogy, Ehrlich ('16) grew several organisms, including *Willia anomala, Oidium lactis, Pichia farinosa, Penicillium glaucum*, and *Aspergillus niger*, on varying concentrations of alkaloids in the presence of various quantities of alcohol, or of invert sugar as carbon sources. Among the alkaloids tried were: cocaine, brucine, morphine, chinchonine, and nicotine. Determining the dry weights of the organisms (where possible) and the N in the mat, and observing the odor and appearance of the culture, he came to the conclusion that compounds possessing an easily split-off N group, as the piperidine group, are more readily assimilable than others. Nicotine, for example, having the easily detached pyrrolidine ring, is better than brucine, morphine, and others in which the N group is held more closely. The molds and bacteria of mixed cultures utilized more of the alkaloids than did pure cultures of individual organisms.

Like Brenner ('11), Puriewitsch ('12) reverts to the view of Czapek that α-amino acids serve directly as materials for protein synthesis and are therefore the most favorable N sources. This investigator attempted to clarify the problem of protein synthesis by determining the energy required for the assimilation of the different nitrogenous compounds. The energy was estimated by measuring the CO_2 evolved per unit of dry weight of the fungus produced. *Aspergillus niger* was the organism employed. The CO_2 was swept out by a stream of air into tubes containing KOH and weighed. Used with dextrose, the amino acids, methyl urea, KSCN, acetamide, urea, and methylamine gave a low ratio $\frac{CO_2}{\text{dry wt.}}$; while KNO_3, ethylamine, phenylurea, guanidine, protein, and peptone gave higher ratios, showing that they required more energy for their assimilation. Ammonium salts occupied about a middle position. Some of the N compounds were also tried with malic, succinic, and tartaric acids as carbon sources in the place of glucose. Although the ratios obtained were somewhat higher than with the sugar, the same general order of assimilability of the N compounds was obtained. However, with malic acid the NH_4 salts were superior to glycine, and KNO_3 almost as good. With respect to the C sources the ratio increased in this order: dextrose, succinic acid, malic acid, and tartaric acid. It is difficult to understand the interpretation of Puriewitsch and his inclination towards Czapek's view, when one examines his data for peptone, which gave a high ratio for all C sources.

Dox and Maynard ('12) cultured *Aspergillus niger* and *Penicillium expansum* in a liquid medium having sucrose and ammonium acid tartrate as the C and N sources, and at the end of each week made determinations of the total and ammonia N of the medium. They found that both forms of N decreased rapidly during the first week of growth, and then during the next 5 weeks increased to a constant quantity. The N retained in the mycelium after this equilibrium had been established was thought to be "some chitin-like substance or glucosamine complex which does not undergo autolytic change." Similar results were obtained when KNO_3 was the N source. Dox ('13) continued this work with

A. niger, making qualitative tests for the presence of sugar and noting the effects on N excretion of replacing the medium weekly by water or by a 2 per cent sucrose solution. The determinations showed that autolysis of this fungus is largely due to the exhaustion of carbohydrate from the medium, because a removal of the autolytic products and substitution of distilled H_2O increased the rate of autolysis, and replacement of the culture solution by sugar solution lessened the rate to one-half that of undisturbed cultures and to one-third that of the cultures in which distilled H_2O replaced the medium.

Waterman ('13), by shaking for 2 hours, a dried, living mat of *Aspergillus niger* with a nutrient solution containing 2 per cent glucose, removing and washing the mat, then boiling for 10 minutes in H_2O and testing the extract with Fehling's, showed that no glucose as such had entered the mold. This was interpreted as showing that the protoplasm of the mold behaved as a semi-permeable membrane toward the glucose, and that adsorption was of no consequence in the accumulation of nutrients. His tables of data show that he determined qualitatively with Nessler's reagent and diphenylamin-sulphuric acid the ammonia present or developed in the culture solution; in some instances this was determined quantitatively with the same reagents. The total nitrogen fixed in the mold and the percentage of sugar assimilated were determined quantitatively, but the methods used were not described. It is assumed that Kjeldahlization, for the N, and some method with Fehling's solution, for the glucose, were employed. Waterman believed that his results showed that "ammonia is a normal excretion product in the metabolism of *Aspergillus niger*" and that this fungus "is able to reduce nitrate to ammonia." A young culture, in which the N content was 2 to 2½ times that of a mature mat, was thoroughly washed and the fungous mat then boiled in water. The extract contained no trace of the inorganic salts originally added to the nutrient, showing that the N salt assimilated by the fungus was quickly changed into another form, and adsorption has little influence on the nutrition. Waterman's other results are given in his own words.

"1. The nitrogen fixed in the mature mould is proportional to the

plastic equivalent of the carbon independently of the nature of the carbon as well as of that of the nitrogen.

"2. The nitrogen number, by which is meant the nitrogen per 100 parts of weight of assimilated carbon, lowers with time; for a mature mould it is about 2 (glucose or levulose as source of carbon).

"3. The metabolism of nitrogen has much resemblance to that of the carbon.

"a. An accumulation of carbon is combined with a high nitrogen number; inversely the mature mould has a low nitrogen number.

"b. The nature of the metabolism of the nitrogen does not change under the influence of many factors; neither is this the case with the carbon.

"c. The velocity of the metabolism is subject to great changes.

"d. The same factors that accelerate the metabolism of the carbon also furthers that of the nitrogen.

"e. Substitution of rubidium for potassium is of little influence on the metabolism of nitrogen.

"4. The nature of the metabolism of the nitrogen is independent of the source of nitrogen. At first the nitrogen number is high, then it decreases whilst the freed nitrogen returns into the nutrient solution as ammonia. This is proved for the cases when ammonium nitrate, ammonium chlorid, or potassium nitrate, is given as N food. *Aspergillus niger*, thus, reduces nitrates to ammonia but not to free nitrogen. Only in the culture tubes with a deficiency of nitrogen as to the quantity of carbon, no ammonia can return into the solution as it is directly used for the production of new cells.

"5. In cases of a deficiency of N no fixation of atmospheric nitrogen could be observed."

Zaleski and Israilsky ('14), working with yeasts, found that single amino acids stood below NH_4 salts in point of assimilability. This, they explained, was comprehensible when one considered that yeast cannot build protein directly out of a single amino acid, but must first deamidize it to obtain N for other groups of the protein molecule. Asparagin was superior to NH_4 salts because the acid amide part of the molecule is readily deamidized and the N group thus obtained is first used in the formation of amino acids and then coupled directly with the liberated aspartic acid to form the protein chain. Aspartic acid and NH_4 salts mixed gave as good growth as asparagin; but, as shown by the fact that the amide group of the asparagin protected added NH_4 salts, the amide group was found superior to NH_4. The best source of N was that found in autolyzed yeast because, they explained, the suitable amino acids were linked directly to form protein. Where asparagin was added to

the autolysate it was found that yeast consumes only about 20 per cent of the amide group, whereas 80 per cent of the amino acid part was used in the synthesis. The description of the methods employed is very indefinite. The fermenting fluid was shaken to obtain a homogeneous mixture and then pipetted off. Protein N was estimated by Stutzer's method and by precipitation with iron acetate. In experiments 5 to 10 total and NH_4.N were determined and in experiment 11 the N of the amide group, but the methods are not given. The work of Ehrlich was criticized on the basis of the absence of NH_3 in the medium.

In an interesting investigation with *Aspergillus niger* in which $(NH_4)_2SO_4$ was used in conjunction with various amino acids, peptone, autolysate, and amino acid mixtures, Zaleski and Pjukow ('14), by determining the total and NH_3.N of the culture fluid, obtained satisfactory results. Knowing the original N they computed from these determinations the kind and amount of N consumed. $(NH_4)_2SO_4$ was utilized to a greater extent than single amino acids in mixtures of the two, but the NH_4 salt, as shown by consumption, was not so good as mixtures of the amino acids or the fungous autolysate, when mixed with these organic sources of N. The order of the serviceability of single amino acids used with $(NH_4)_2SO_4$ was phenylamine, leucine, glycine, alanine, aspartic acid; histidine was not utilized. The fact that $(NH_4)_2SO_4$ was used even in the amino acid mixtures showed that the fungus synthesized other amino acids. The decomposition of the various amino acids proceeded at different rates which were proportional to their utility for this organism. The relative usefulness of the NH_4 salts and amino acids could be varied in several ways: (1) by varying the acid radical to which the (NH_4) was joined, (2) by changing the carbon source; with a less available carbon source the amino acid was more utilized, (3) by the addition of a stimulant, as $ZnSO_4$, which lessened the consumption of the amino acid by promoting a more economic consumption of the sugar. Glycine in the presence of CH_3.$COONH_4$ was not used. The addition of $CaCO_3$ to the nutrient containing $(NH_4)_2SO_4$ and alanine decreased the use of the alanine, and this was explained by saying that the carbonate neutralized the acid produced in growth and produced an alkaline

reaction adverse to the decomposition of the amino acid. In the presence of a good C source NH_4 was found a better N source for the fungus than single amino acids, but a suitable mixture of amino acids was better than NH_4.

In his severe criticism of the opinions of Czapek, Puriewitsch, Brenner, and others on the direct usability of amino acids, Boas ('18) pointed out the fallacy of attempting to judge the comparative assimilability of N nutrients under the widely varying conditions of experimentation employed by those workers. For example, a hydrochloride of guanidine could not satisfactorily be compared with an amino acid because of the liberation from the former under the action of a fungus of an abundance of the inhibiting acid, HCl. He emphasized the necessity for comparing compounds at the same hydrion concentration; for example, in the comparison of asparagin and $(NH_4)_2SO_4$, the production of soluble starch and delay in sporulation observed with the latter compound, and which indicated an abnormal metabolism, resulted from or was conditioned by the liberation of H_2SO_4. Czapek, in his choice of incubation periods of 21 days or longer, it was pointed out, entirely ignored the effects of proteolysis on the fungous crops. Boas then repeated Czapek's experiment with guanidine, urea, and biuret, making weighings of the fungous crop, at different time intervals and obtained a reversal of the order of assimilability. The free acid liberated from the guanidine-HCl, it was thought, inhibited autolysis so that a long incubation period eventually gave a larger crop. Puriewitsch was also criticized on the same basis. Boas explained that the energy quotient would be a valuable index of the assimilability of N compounds, if due regard were given to the use of a variety of C sources, to the hydrion concentration of the medium, to the formation of soluble starch, to the effects of proteolysis, and to the influence of outer conditions, as temperature, light, humidity, and aeration. He then gave corrective experiments. Mannite, glycerine, malic acid, and quinic acid were employed as C sources, because, as he showed, the liberation of acid from the ammonium salts of the strong acids did not upset the carbon metabolism of the fungus when these compounds were used; soluble starch was not formed and there was no delay

in fruiting. In connection with these carbon compounds referred to, $(NH_4)_2SO_4$ and $(NH_4)_3PO_4$ were found superior to asparagin and glycine as N sources. The energy requirements for the amino acids were higher because of the necessary deamidization. The energy quotient for asparagin was raised by the addition of free H_2SO_4 to the culture fluid.

In his 1919 work Boas showed also that the rate of absorption of a compound was proportional to its extent of dissociation; for example, the highly dissociated NH_4 salts of strong mineral acids were utilized before glycine, acid amides, and peptones. This relation held in spite of strong acid formation and the consequent production of soluble starch and inhibition of sporulation. His 1922 article gave data showing that on media containing respectively, levulose, sucrose, dextrose, maltose, and galactose, decreasingly in order of the sugars listed, *Aspergillus niger* was capable of forming soluble starch. There appeared to be a close connection between diastase formation, sporulation, and hydrion concentration. In contrast to the behavior of *Aspergillus niger*, *A. Oryzae* on maltose caused soluble starch formation but on levulose produced none.

Waksman ('18, '19, '20), in his extensive studies with soil organisms, particularly the *Actinomyces*, gave due attention to the nitrogen relations. His 1918 work with several species of *Aspergillus*, *Citromyces*, *Penicillium*, and *Actinomyces*, and *Bacterium mycoides* showed that such rapidly growing molds as *Aspergillus* and *Penicillium* on Czapek's solution, with peptone or casein as the N source, produced an abundance of ammonia which gradually increased with the duration of incubation; amino nitrogen, on the other hand, tended to decrease, indicating use by the organisms. The slower-growing *Actinomyces* and *Bacterium mycoides* brought about a large accumulation of amino nitrogen and a relatively small accumulation of ammonia. Another group, represented by *Citromyces* spp., favored the accumulation of both forms of N in relatively large amounts. In the work with *Aspergillus niger* it was shown that this accumulation of NH_3 took place both in the absence and presence of sugar, but the sugar depressed the rate of production. In the presence of sugar the curve for NH_3 accumulation followed remarkably the

theoretical curve for autocatalysis. The relation of ammonia production to carbohydrate present was further shown by the culture of *A. niger* on media containing varying percentages of asparagin and the same concentration of sugar. On media having 0.5, 1.0, and 2.5 per cent asparagin the organism after reaching its maximum growth (in about 3 days) rapidly decomposed the amide-amino acid into ammonia nitrogen; on the other hand, where only little asparagin was present and the amount of sugar remained the same, that is, relatively large in comparison, little or no ammonia was produced and the amount of NH_3.N simply decreased.

Making further use of the van Slyke "micro" method for amino N, Waksman ('18) made a study of the proteolytic enzymes of several soil fungi, *Aspergillus niger* being employed in most of the work. He showed that *Penicillium chrysogenum* and *Actinomyces* sp. 101, which had been found to favor the production of more amino than ammonia N when grown in a peptone solution, produced strongly proteolytic enzymes, whereas the organisms, as several species of *Aspergillus*, which produced more ammonia than amino N, formed weaker enzymes. Compared with the proteolytic enzymes of animal origin, the fungous enzymes were found to differ in being less limited by hydrion concentration (P_H values were not determined; reactions were indicated by litmus and phenolphthalein), by having a lower optimum temperature, by not being precipitated by safranin, and in being able to pass a Pasteur-Chamberland filter. In its production of enzymes *Aspergillus niger* was not influenced by the sugar content of the medium. The activity of both exo- and endo-proteoclastic enzymes produced by the fungi in peptone-containing media was greater than that of the enzymes produced in nutrients containing no peptone. The fast-growing *Aspergillus niger* produced its most active enzymes during the first 3 days of incubation, whereas the slower-growing *A. ochraceus* increased in proteoclastic activity up to the eighteenth day of incubation, after which there was a decline. He attempted an explanation on the grounds that *A. niger*, reaching its maximum growth in about 3 days, during this time necessarily produced strongly acting enzymes and also acids, which acted injuriously

on the enzymes. The greatest enzymatic activity for *A. niger* was therefore observed during the first 3 days of incubation. *Aspergillus ochraceus*, on the other hand, was considered to produce no injurious acids, and its enzymes increased in activity until the inhibiting autolytic substances began to be formed. The exo- and endo-enzymes were similar in their action. Deamidizing enzymes were indicated by the detection of small amounts of ammonia in the treated peptone and casein.

In the work on the N metabolism of the *Actinomyces* Waksman and Curtis ('16) and Waksman ('18, '19, '20) made use of Nessler's reagent for qualitative ammonia tests and the aeration method for quantitative determinations; amino N was estimated by the "micro" method of van Slyke, nitrites by the colorimetric method of Griescz, and active acidity with Clark and Lub's indicators. The results may be summed thus. Nearly all *Actinomycetes* were capable of liquefying gelatine and many could haemolyze blood agar and liquefy blood serum; in other words many of these forms have a strong proteoclastic power. This process was followed in protein solutions by means of amino acid determinations. The amount of splitting was directly proportional to the extent of growth. Although on long incubation a considerable quantity of NH_3 accumulated, the production of NH_3 from amino acids and proteins by *Actinomyces* was not considered characteristic. None could fix elementary N_2. With a suitable C source nearly all species could reduce nitrates to nitrites. In the order of assimilability as sources of N stood, speaking broadly, proteins and amino acids, nitrates, nitrites, amides. With glycerine as a carbon source NH_4 salts were the poorest sources of N; but with dextrose both NH_4 salts and amides were well assimilated if the medium did not become too acid.

Waksman and Joffe ('19) reviewed some of the papers on the application of hydrion determinations to bacteriological work; they then gave the results of investigations on the effect of *Actinomycetes* on the reaction of Czapek's solution containing various C and N sources. The colorimetric method was used. It was shown by growing the organisms on $NaNO_3$ and varying the C source that the *Actinomycetes*, unlike many bacteria, are not producers of acid from carbohydrates; in fact under the con-

ditions the medium showed a tendency toward alkalinity. His explanation was that the nitrate, under the influence of these organisms, is reduced to nitrite and the liberated oxygen is united with the reducing H to form hydroxyl ions which reduce the hydrion concentration of the medium. With the conditions reversed, employing constant C source (glycerine) and different N sources, the change in the reaction of the medium was shown to be due to the N source, $(NH_4)_2SO_4$ medium, for example, rising in hydrion concentration from P_H 5.8 to 4.2. The change of P_H in protein and amino acid media was found very variable, depending on the species of the organism cultured, the original P_H, and the C source. An available C source in a protein medium favored the production of an acid reaction; this was thought to be due to the effect of the carbohydrate on the N metabolism and not to the formation of acids from the C source. Some species always increased the $[\overset{+}{H}]$ in such media, while others lessened it. Leucine nearly always favored acidity. The $[\overset{+}{H}]$ of all media was shifted toward the optimum by these organisms.

Molliard ('18) studied the rate of consumption by *Sterigmatocystis nigra* of glucose and levulose, resulting from the inversion of sucrose, in a modified Raulin's solution. Sucrose was added with HCl in 2 different concentrations (290 mgm. and 310 mgm. HCl per 150 ml. of medium) for the 2 series run. The increase in acidity lowered the harvest of fungus, but increased the consumption of sugar. The determinations made at 21 days showed that all the glucose, but only about one-sixth of the levulose, had disappeared. In his 1920 work on the effect of reaction on the liberation of CO_2 from cultures of the same fungus Molliard varied the acidity with H_2SO_4 and Na_2CO_3, and recorded the reaction in terms of normality of acidity or alkalinity. Determinations of active acidity were not made. It was found that the amount of CO_2 set free in respiration increased rapidly from 0.1 *N* alkalinity to a maximum at 0.02 *N* alkalinity, then diminished slowly to 0.06 *N* alkalinity,—beyond this very rapidly. Oxalic acid was said not to be produced in the acidity range greater than 0.02 *N* H_2SO_4, but steadily increased in production as the medium was made more alkaline, reaching a maximum at 0.06 *N* alkalinity.

Raistrick and Clark ('19) determined the relation of various carbon sources (organic acids) to oxalic acid formation by *Aspergillus niger*. Oxalic acid was determined by precipitation as calcium oxalate and titration with permanganate. The results are summarized:

1. Four carbon, dibasic acids gave good growth and a good yield of $(COOH)_2$.
2. Four carbon, monobasic acids gave almost no growth and $(COOH)_2$.
3. Three carbon acids gave very good growth, but little or no $(COOH)_2$.
4. Two carbon acids, as acetic, gave good growth and yield of $(COOH)_2$, while glycollic and glyoxalic gave but fair growth and no $(COOH)_2$.
5. The one carbon acid, HCOOH, gave but fair growth and no oxalic acid.

A theory was given to account for the formation of $(COOH)_2$, citric, and fumaric acids from sugars from other organic acids. In the case of $(COOH)_2$ production from sugar, diketo adipic acid is formed which is hydrolyzed to acetic and oxalic acids, the CH_3COOH then being also oxidized to $(COOH)_2$. With the organic acids as C sources, oxalacetic acid is formed by hydrolysis or oxidation or both, depending upon the organic acid used, and this breaks to form $(COOH)_2$ and CH_3COOH. The production of citric and fumaric acids from sugar was supposed to proceed through oxalacetic acid. The sources of the inorganic N (NH_4 or NO_3 ions) were found to have no influence on the quantity of $(COOH)_2$ formed.

Lampitt ('19) presented data relative to the N metabolism of bread yeast. He determined total N by Kjeldahlization, NH_3 by distillation with MgO *in vacuo*, and sugar by Bertrand's method. Deamidization of amino acids was not studied by determinations of amino nitrogen but by the ammonia produced. It was found that the removal of N from the nutrient was proportional to the yeast present; and, as determined by counts and N determinations, the greater the rapidity of budding during active fermentation the greater the amount of N assimilated by each cell. However, active reproduction was found in some

cases to result in a lowering of the N coefficient of the yeast, even in the presence of an abundance of N. The final N coefficient of the yeast was found to be independent of the initial coefficient for the particular conditions of reproduction.

A non-volatile acid, thought to be malic, resulted from the action of yeast on asparagin. Malic acid itself was not fermentable; but its NH_4 salt was entirely decomposed, producing C_2H_5OH. Propionic acid, which, according to Effront, results from the action of amidases on asparagin, was not fermented and its NH_4 salt only slightly attacked. Fermentation was found to be necessary to the assimilation of N, yet the two processes were not proportional, for deamidization was retarded during excessive zymatic action and sometimes continued after this had ceased. Excretion of N into the medium also was found to be conditioned by fermentation, but not proportional to it; it was, however, directly proportional to the sugar present. The process took place simultaneously with N assimilation, indicating that excretion was interrelated with the life of the cell; and the N excreted was shown to be utilizable under certain conditions.

Iwanoff ('21) pointed out the defects of the Stutzer method, as used in the determination of the protein N, of fermenting fluids; nitrogenous bodies having no protein character were found to be precipitated by the $Cu(OH)_2$. These bodies were huminose in character and resulted from the reaction of sugar with an N-containing substance coming from the yeast. These huminose compounds plus alcohol and the other products of fermentation arrested protein decomposition more than did alcohol of the strength present in the fermenting liquid, but it was thought that the acids present were largely responsible for the difference. Alcohol, however, was found to be the chief inhibitor of all the fermentation products, 7 per cent strongly inhibiting the protein decomposition. The addition of KH_2PO_4 tended to annul this. As a possible explanation of Iwanoff's huminose compounds Gortner and Holm ('17) have shown that the dark nitrogenous substance, "humin" N, formed during the acid hydrolysis of proteins is due to the action of an aldehyde on the indole group of tryptophane.

Haenseler ('21) found that the yield in dry weight of *Aspergillus*

niger was proportional to the amount of nitrate and also to the concentration of the sugar of the culture medium.

The action of nitrate on lower organisms was studied by Böttger ('21). Toxicity appeared at a certain concentration of the nitrate, above which it became increasingly inhibitive until the organisms were killed. The initial point of toxicity varied with the other components of the nutrient. The different functions, such as growth, enzymatic activity, and sporulation, exhibited toxic response at different concentrations of the nitrate. The specific nature of the toxic property was not determined, but it was thought to be nutritive as well as physical (osmosis). All nitrates were found toxic regardless of the cation. Waterman ('18) gave a series of tables indicating the influence of KNO_3 on the rapidity of growth of *Aspergillus glaucus*. Kossowicz ('12) demonstrated the poisonous effect of $CaCN_2$ on 10 different fungi.

Self-poisoning of fungi might be briefly mentioned here, as the process is closely related to N metabolism. Uhlenhaut ('11) grew species of *Mucor* on media containing the glucoside, amygdalin, and found that growth was soon inhibited. This inhibition was ascribed to the accumulation of benzene cyanhydrine which imparted an easily recognizable odor to the solution. Where fungi capable of utilizing cyanhydrine were cultivated with *Mucor*, the latter made a much more abundant growth than in pure culture. Amygdalin was little used in the presence of other C sources. Wehmer ('13) noted self-poisoning of *Penicillium variabile* on media with $(NH_4)_2SO_4$ as the N source. Boas ('19), in a former article, had shown that a *Cladosporium* in media having urea as the N source produced such large quantities of NH_3 that the fungus was soon killed; amines, produced proteolytically, were also thought to be instrumental in the death of the fungus. *Aspergillus niger*, on a solution containing 2 per cent urea, 5 per cent maltose, and the usual mineral salts, quickly changed the reaction of the medium to strongly alkaline (P_H 7.5–8.3). A strong odor of ammonia and amines was evident, the NH_3 neutralizing the oxalic acid produced from the sugar. The fungus was killed in from 7 to 9 days on this medium, as it was also on that containing various amounts of maltose, dextrose plus

maltose, and sucrose with urea and acetamide in different proportions. Other fungi, including *Botrytis cinerea* and an *Oidium*, behaved quite differently under these conditions, remaining alive for months and not producing excess NH_3.

Terroine, Wurmser, and Montaine ('22) investigated the total N content of *Aspergillus niger* grown under various conditions. With the development of the fungus the N percentage of the mat was found to decrease; this was not influenced by the concentration of the N of the medium. In the first part of the incubation, when the N content of the medium was 0.5 per cent $(NH_4)_2SO_4$, an increase in the sucrose or glucose was followed by an increase in the N fixed; in the latter part, by a decrease. Urea or $NaNO_3$ substituted for $(NH_4)_2SO_4$ resulted in a slight fall in the percentage of fungous N, but peptone and guanidine caused a decline respectively of 18.3 and 45.0 per cent. Xylose and arabinose with $(NH_4)_2SO_4$ served in this respect exactly as glucose or sucrose, but galactose caused a reduction of 21 per cent. The mycelium of a normal culture washed and placed in Czapek's solution minus N lost over 50 per cent of its N in 5 days' incubation at 37° C.

Butkewitsch ('22) grew *Aspergillus niger* and *Citromyces* spp. on culture solutions containing the usual minerals plus 0.2 per cent $ZnSO_4$ and respectively 2.5, 5.0, 10.0, and 20.0 per cent peptone and determined the oxalic acid, the ammonia, and likewise the dry weight of fungus produced in 10-, 20-, 30-, and 40-day periods of incubation. Hydrion concentration determinations were not made, litmus being employed to indicate the reaction. Ammonia was estimated by distillation with MgO or CaO *in vacuo*, and $(COOH)_2$ by precipitation as calcium oxalate and titration with $KMnO_4$. The results were interpreted as showing that the proportion of $(COOH)_2$ to NH_3 approached that of neutral $(COONH_4)_2$, but generally showing a predominance of NH_3. The younger cultures were acid because in addition to $(COOH)_2$ they contained the acids freed in the deamidization of the amino acids by the fungus; the older cultures were alkaline because of the excess NH_3 not bound with acids, but as $(NH_4)_2CO_3$. Most of the NH_3 was produced in the period of mat development, 90 per cent of it being formed in the first

10 days; the ratio $\frac{\text{dry wt.}}{NH_3.N}$ was consequently greatest in this period and decreased as the culture aged. This diminution of the ratio $\frac{\text{dry wt.}}{NH_3.N}$ was found to correspond to the peptone content of the medium; the greater the initial growth the greater the ultimate decrease in weight. Rise of temperature decreased this fraction. Age and temperature determined also the economic coefficient of the N-free complex of the peptone. Zinc salts had no effect on the utilization of peptone, so that the increase in harvest obtained with media containing carbohydrate as a C source to which Zn had been added was explained as due to stimulation of carbon metabolism. *Citromyces* behaved similarly to *Aspergillus*, but produced a slightly higher percentage of NH_3.

Boncquet ('16) found nitrites and NH_3 in material from "mosaic" tobacco and potato, and from beets affected with "curly leaf." He ascribed the presence of these unusual nitrogenous forms to the "reducing power of the internal bacterial flora." Jodidi, Moulton, and Markley ('20) ran parallel nitrogen analyses with normal and "mosaic" spinach and found remarkable differences. Diseased plants had a smaller percentage of total protein, nitrate, acid amide, mono- and di-amino N, but a slightly higher percentage of NH_3 than normal plants; nitrites were found only in the diseased plants. Obtaining similar results with material from normal cabbage and that affected by the so-called "mosaic disease" they believed they were warranted in cataloging the disease as "mosaic." Klotz ('21), using the same methods, obtained similar analytical data working with celery leaves affected with the *Cercospora* blight; later it was found that celery leaves diseased with *Septoria* blight showed a similar chemical picture. In the latter case more modern methods were used.

Experimental

Materials and Methods

Three fungi, viz., *Aspergillus niger*, *Sphaeropsis malorum*, and *Diplodia natalensis* were used in the investigations reported in this paper. Work with *Phoma Betae*, a form particularly in-

teresting because of its capacity for fixing free N_2 (Duggar and Davis, '16), is now under way. The investigation of other forms, including yeasts and bacteria, is contemplated.

The organisms were grown on Duggar's solution for fungi,[1] the nitrogen source being varied to give 5 different kinds of media. The solution consisted of the following chemicals per 50 ml.

		ml.
Dextrose	0.5 *M* solution	25
KH_2PO_4	0.25 *M* solution	10
$MgSO_4.7H_2O$	0.1 *M* solution	5
N source	*M* solution	10

In addition to the above 0.1 ml. of 0.001 *M* $FeCl_3.6H_2O$ was added to each culture. The KH_2PO_4, $MgSO_4.7H_2O$, and $FeCl_3.6H_2O$ were Merck's highest purity grade. The inorganic N sources used were KNO_3 (Merck's reagent crystals), NH_4NO_3 (H. P. Merck), and $(NH_4)_2SO_4$ (Merck's reagent grade). The salts were dissolved in warm distilled water (P_H 5.3), cooled to 20° C., made to volume, placed at a temperature of 7° C. for 3 days, and filtered. The stock solutions were kept in the dark at a temperature of 7° C.; before use they were brought to room temperature (20–23° C.). "Bacto" dextrose, having less than 1.0 per cent H_2O, usually about 0.25 per cent, was used. "Bacto" peptone was used in the concentration of 11.65 per cent throughout the work. The glucose and peptone solutions were made up just before using, thus necessitating but one sterilization which, for the peptone, was important in keeping comparable the amount of hydrolysis. The 5 different media are characterized in the data by the N source, namely, "peptone plus" (No. 1), "peptone minus" (No. 2), $(NH_4)_2SO_4$ (No. 3), NH_4NO_3 (No. 4), and KNO_3 (No. 5). The media numbered 1, 3, 4, and 5 have glucose as a carbon source, but in No. 2 the peptone serves as the source of both C and N, the 25 ml. of dextrose being replaced by 25 ml. H_2O.

Quantities of 50 ml. of the media were placed in 300 ml. Erlenmeyer flasks. The flasks were in all cases first thoroughly cleaned by washing in warm tap-water and cleaning solution, followed by several rinsings of hot tap-water and finally with distilled water.

[1] Being published.

For sterilization the media were autoclaved at 16 pounds steam pressure for 15 minutes. The sterile flasks were carried from the autoclave immediately to a thoroughly steamed culture room and inoculated when cool.

Three methods of inoculation were used in the course of the work. In the first and second series with *Aspergillus niger* plantings of the fungus were effected by wetting a platinum loop in the sterile media, placing it on the aerial spores of a potato glucose agar slant culture, and then transferring the loopful of spores to the medium. In the third series with this fungus a spore suspension was made by pouring 10 ml. of sterile distilled water on an agar slant culture, loosening the spores into the H_2O by means of a platinum needle and pouring the suspension into 100 ml. of H_2O in an Erlenmeyer flask. The flask was thoroughly shaken to obtain a uniform distribution of the dilute suspension and let stand over night to aid in wetting the spores. After shaking again the inoculum was taken up by means of a 25-ml. graduated pipette and 0.5-ml. portions placed into each flask of medium. With the *Sphaeropsis* and *Diplodia*, foims which did not sporulate in culture, uniform inoculations were obtained by planting the culture media with small (5 mm.) discs of inoculum from giant petri dish cultures on a thin (2 mm.) layer of potato-glucose (or sucrose) agar. As checks, several flasks of each medium were planted immediately before sterilization with the same amount of inoculum. All cultures in all series were incubated at 28° C. in darkness, a large incubator with double doors and water-jacket being used.

At the intervals shown in the following tables 5 cultures of each medium were analyzed, determinations being made of the dry weight and percentage N of the fungous crop, and hydrion concentration, sugar, NH_3 plus NH_4.N, total N, NO_3.N, NO_2.N, total amino N, acid amide N, and peptid N of the culture fluid. Near the end of each experiment entire cultures, including the mat and medium, were Kjeldahlized to ascertain the loss or gain of N and the presence or absence of the capacity of the fungi for N fixation under the conditions employed. The 5 culture solutions of each medium were filtered into a 500-ml. volumetric flask and the mats thoroughly washed, the wash water being

collected in the volumetric flask with the culture solutions. Aliquot portions of the filtrate and washings, which were made to volume and thoroughly mixed, were used for the determinations enumerated above.

The fungous mats were dried at 100–105° C. in an electric oven, cooled in a desiccator, and weighed to the nearest milligram on a "chainomatic" balance.

The active acidity of the culture solution was determined colorimetrically by employing the indicators and buffer solutions suggested by Clark and Lubs ('17) and Clark ('20). A comparator blank was used throughout. Near the ends of the indcator ranges it was helpful to make use of the colorimeter (Duggar and Dodge, '19), as this instrument extends the range and usefulness of an indicator. The micro Duboscq instrument can be applied very satisfactorily to this work by following these measurements. Two ml. of unknown plus 2 drops of indicator should be placed in the lower right cup, and in the cylinder above this .625 ml. of H_2O. In the left cup and cylinder should be placed respectively the corresponding quantities of known buffer plus indicator and compensating unknown solution. The readings are made at the 16.5th graduation in order to obtain like columns of colored solution and compensating solution.

Various methods for determining reducing sugars were tried. The results reported in the first series with *Aspergillus niger* were obtained by direct titration of the culture solution against Fehling's solution, the various indicators used to determine the absence of Cu and end point of the titration being tried. The 1920 iodometric method of Shaffer and Hartmann ('20) was found most satisfactory as it is accurate and rapid. The Fehling's modification was used throughout for "macro" quantities of sugar, as closer checks could be obtained with this than with the carbonate-citrate reagent. The advantage of the latter in having the chemicals combined in a single solution is outweighed by the increased cost and by the danger of loss of material from foaming when the solution is acidified at the end of the reduction. The "micro" method described in that paper was used in the second and third series with *Aspergillus niger* when the concentration of the glucose became sufficiently low; it was used in

subsequent series simply to ascertain the absence of the reducing sugar. Applied to the determination of dextrose in such media the "micro" method was found to give such variable results at different dilutions that it was entirely unsatisfactory for general use. Many of the following, very helpful suggestions were obtained from members of the staff at the Washington University Biochemical Department and are here given because they are not included in the paper. The "iodate-iodide" solution should be made slightly alkaline by the addition per liter of 0.4 ml. saturated NaOH solution; this prevents the formation of hydriodic acid. The standard thiosulphate solution is made permanent in the same way.

The thiosulphate solution is readily standardized by titration against a 0.1 *N* potassium biniodate solution (93.24958 gm. $KH(IO_3)_2$ per liter make 0.1 normality). To 50 ml. of the biniodate are added 3 gm. KI (dissolved in 25 ml. H_2O), and 10 ml. of an approximately 5 *N* H_2SO_4 or HCl. The excess acid and iodide with the biniodate liberate iodine equivalent to exactly 50 ml. of a 0.1 *N* solution, according to the following equation.

$$KH(IO_3)_2 + 10\,KI + 11\,HCl - 6\,I_2 + 11\,KCl + 6\,H_2O$$

Titrate against the thiosulphate, using 2–3 ml. of starch solution as an indicator when the iodine color becomes faint.

Starch indicator solution made from arrowroot starch is preferable to a solution made from soluble starch because the latter deteriorates more rapidly under septic conditions and then fails to give the starch-iodine blue. Arrowroot starch solution kept for several weeks still retained its usefulness as an indicator. Of course any starch may be used, but the arrowroot is preferable because it is a standard, easily obtainable product. Two grams of the starch were shaken in 100 ml. cold water and poured into 100 ml. of boiling water; one ml. of 5 *N* H_2SO_4 was added and boiled about a minute.

The reduction is most uniformly effected by heating over a direct flame; an asbestos mat with a 2-inch circular hole is serviceable. A carrier, very handy for removing the hot flasks, is easily made by cutting away the side of a small, cylindrical wire test-tube basket, making an opening large enough to admit a 300-ml. Erlenmeyer. To cool, flasks are set in a shallow dish, as an evaporation dish, under running water.

The small quantity of reducing substances, other than dextrose, found in the peptone media could not be satisfactorily removed by precipitation with sodium tungstate as described for milk in Shaffer & Hartmann's ('20) paper. Lloyd's reagent,[1] as employed by Folin and Bergland ('22) (in the determination of sugars in urine), would adsorb some but not all of these reducing substances. Accordingly, in the later determinations the small quantity of copper reduced by the peptone was estimated by running blank determinations on the "peptone minus" (No. 2) medium, and subtracting this result from that given by the "peptone plus" (No. 1) medium.

The principle of the Folin ('05) aeration method was used in obtaining the results for ammonia nitrogen (see Shaffer, '03). To make the solution strongly alkaline to phenolphthalein 0.1 ml. of a saturated NaOH solution was used for each 5 ml. of unknown. The strong alkali is preferable to Na_2CO_3 because it assures the freeing of the NH_3 from the small amount of $MgNH_4PO_4.6H_2O$ that may form in the alkaline solution (see page 347). Tubes were connected in series so that 7 aerations could be made at the same time. Folin tubes with perforated bulbs were used throughout the apparatus for aerating the unknown and for delivery of the NH_3 into the receiving liquid. By means of a filter pump the solutions were aerated for 18–24 hours at the rate of approximately 50 liters of air per hour. In this, as well as in the determinations for total and nitrate N, 4 per cent boric acid was used to collect the NH_3. As in the Scales and Harrison ('20) method for total N the collected NH_3 was titrated directly against standard H_2SO_4, brom-phenol-blue being used as the indicator. This was compared with the usual method of collection in a standardized acid and titration back against standard alkali; the boric acid modification is absolutely as accurate as the old method and dispenses with the necessity of a standard alkali (see also Paul and Berry, '21, and Spears, '21).

Total N, including nitrates, was estimated by the method of Davisson and Parsons ('19). In this the nitrates are first reduced to NH_3 by means of Devarda's alloy in alkaline solution, the NH_3 being caught in strong H_2SO_4 (7 parts H_2SO_4 to 1 part

[1] A hydrated aluminum silicate obtained from Eli Lilly & Co., Indianapolis, Ind.

H_2O). The $(NH_4)_2SO_4$ in the acid was then returned to the flask and digestion effected as in the Kjeldahl-Gunning method, 5 grams of powdered K_2SO_4 being used to raise the temperature of the digesting liquid. Before distillation 25 ml. of 4 per cent K_2S solution were added to precipitate the mercury from mercurammonium compounds. Where the determination did not involve nitrates the Gunning modification of the Kjeldahl method as given on page 7 of 'Official Methods' (Association of Official Agricultural Chemists, '21) was used. Trouble from bumping during distillation was entirely overcome by the addition of approximately a tablespoonful of glass beads and a gram of powdered pumice stone to each flask. In my hands the official Gunning method modified to include the N of nitrates (page 8 of 'Official Methods') was entirely unsatisfactory, even with the utmost precautions; particularly if the "hypo" was added all at once a brownish gas could frequently be seen above the liquid, indicating a loss of N. This was to all appearances overcome by adding the thiosulphate slowly and shaking continuously during the addition, but the results on known solutions of nitrate were very variable. The following show the trend of the results obtained by the official method.

TABLE I

TOTAL NITROGEN INCLUDING NITRATES

	Ml. 1055 *N* H_2SO_4	Mgm. N	Theory
10 ml. of 0.1 *M* KNO_3 solution	9.15	13.530	14.010
5 ml. of 0.1 *M* KNO_3 solution	4.05	5.986	7.005
5 ml. of 0.1 *M* KNO_3 solution	4.15	6.134	7.005
10 ml. of 0.1 *M* NH_4NO_3 solution	17.60	26.010	28.020
5 ml. of 0.1 *M* NH_4NO_3 solution	9.30	13.750	14.010

The method outlined at the beginning of this paragraph gave dependable results and was much more rapidly carried out than the official method.

So far as I have been able to determine, there is no satisfactory method for the determination of nitrate N in the presence of ammonium and other forms of N and organic substances. Scales' ('16) method of reduction with Zn-Cu couple gives accurate results, if great precaution is taken with the couple. However,

the time and trouble required to clean and renew the material and in adding the NaCl and MgO, together with the necessity of running blanks without couple where the solution contained $NH_4.N$, made it inadvisable to use this method. The difficulty of the proper execution of the old gasometric method of Schulze-Tiemann (Emmerling, '12) is recognized by those who have employed it. The method of Strowd ('20) was adopted because of its simplicity and its fair degree of accuracy. In this the nitrates are reduced to NH_3 by means of Devarda's alloy in the presence of alkali, blanks with alkali but no alloy being "run" at the same time under as nearly similar conditions as possible. This did not give dependable results, however, in the presence of dextrose, the sugar in some way holding back some of the nitrate. Accordingly the results obtained with media containing sugar, as given in the following data, are not considered reliable. The results obtained in the third series with *Aspergillus niger* are shown in the graphs (fig. 10), because they indicate relatively the course of nitrate consumption by this fungus; moreover, the sugar being entirely consumed by this organism in 5 days, the results for nitrate carry more weight. In subsequent series the nitrate was determined only after the disappearance of the sugar, and the results given were obtained by subtracting the result for NH_3 from that for total N.

Nitrites were determined in the first series with *Aspergillus niger*, a "micro" modification of the official method (page 22, 'Official Methods'), as applied to water analysis, being developed and used. In the official method the color ratios are obtained by varying, in 1 ml. quantities or multiples thereof, the amount of standard nitrite in the 50 or 100 ml. of H_2O, each ml. of standard containing 0.0001 mgm. of nitrite. The same quantities of reagents are added to each tube of standard and unknown. In the "micro" method the proportions of standard nitrite to 5 ml. water were maintained by use of a pipette that delivered 20 drops per ml. It is seen, therefore, that each drop of standard nitrite in 5 ml. of H_2O corresponds to the 1 ml. of standard nitrite in 100 ml. of H_2O. The standards were made in small, serological test-tubes marked at 5 ml. The same corresponding amounts of the reagents (concentrated HCl diluted 10 times,

sulphanilic acid, and a-naphthylamine-HCl) were then added to the standards and 5-ml. portions of unknowns. One or more drops of the respective reagents may be employed so long as the amounts are constant for each test. The same concentration of HCl as given in the official method is obtained by diluting the C.P. HCl to one-tenth its strength. The solutions are allowed to stand 30 minutes before the color comparisons are made. The standards are made up fresh each time determinations are made. Other comparable ratios for pipettes delivering other than 20 drops per ml. are readily calculated, and a volume other than 5 ml. may be chosen if the proper adjustments are made. The advantages of the "micro" method are the ease of making quickly a large number of standards, the economy in the use of reagents and unknown solution, and the more accurate comparison of the unknowns and standards by use of the comparator block and the Duboscq colorimeter. Turbidity and color is compensated for the same as in the hydrion determinations.

Amino nitrogen determinations were made on the material aerated in the NH_3 estimations by means of the van Slyke "micro" method in which 2 ml. of unknown solution were used. The aeration removes NH_3 and free amides which would interfere, giving abnormally large quantities of free N. Several preliminary experiments showed that an interfering NH_4 salt may be separated from an amino acid in this way. One of these experiments is here given. Two 10-ml. samples of 0.1 *N* solution of $(NH_4)_2SO_4$ were added respectively to two 10-ml. quantities of an approximately 0.1 *N* alanine solution, 1 gm. K_2CO_3 added, and the combined solutions aerated for separation and determination of the NH_3.N. Two 10-ml. samples of 0.1 *N* $(NH_4)_2SO_4$ plus 1 gm. K_2CO_3 were aerated alone, as were 2 similar quantities of alanine solution. Three 10-ml. portions of the $(NH_4)_2SO_4$ solutions were estimated by the Kjeldahl method, as were two 10-ml. aliquots of alanine.

TABLE II

EFFICIENCY OF AERATION METHOD FOR SEPARATION OF (NH_4) AND $(NH_2)N$

	Ml. 1055 *N* H_2SO_4	Mgm. N
1. N from $(NH_4)_2SO_4$ by Kjeldahlization	9.10 9.15 9.15	13.52 13.52
2. N from alanine by Kjeldahlization	9.10 9.10	13.538
3. N from $(NH_4)_2SO_4$ by aeration	9.10 9.10	13.45
4. N from $(NH_4)_2SO_4$-alanine by aeration	8.95 9.05	13.376
5. N from alanine by aeration	—	—
	Ml. N_2	Mgm. NH_2. N
6. Amino N from 0.05 N alanine, 2 ml. Before aeration 24° C.—766 mm. 24° C.—766 mm. 22° C.—766 mm.	 2.33 2.35 2.35	 1.3106 1.3218 1.3348
After aeration 22° C.—766 mm.	2.35 2.34	1.3348 1.329
7. Amino N from alanine $(NH_4)_2SO_4$ After aeration 21° C.—766 mm. 21° C.—766 mm.	 2.338 2.355	 1.335 1.3447

These experiments show that aeration for 18 hours at the rate of 30–50 liters per hour is a satisfactory means of determining NH_3.N, and of separating NH_3 from amino N. Glucose and other constituents of the media were not found to interfere with the completeness of aeration.

The van Slyke "micro" method ('11, '11a, '12, '13, '15), rather than the "macro," was adopted because the apparatus used in the former is much more stable, requires only a fifth of the quantity of reagents, and the modification is as accurate as the original "macro" method. Special precautions must be observed in this determination. A definite period of reaction must be adopted and maintained throughout a given piece of work to obtain comparable results, because the blanks with water as well as the determinations on solutions containing NH_2.N vary with the time. In all the following work 5 minutes

were adopted as the time of reaction. A large number of determinations were made with distilled H_2O and various solutions to study the effect of duration of reaction on the quantity of N_2 evolved. A few determinations with 2 ml. of water are here given:

	Time in minutes	ml. N_2
Temperature 23° C.	3	.08
	5	.085
	5	.09
	8	.11
Barometer 763 mm.	20	.12
	40	.15

The blank appears to vary regularly with the quantity of gas given off by the nitrite. Variations in the blanks are to be expected with different supplies and grades of nitrite and acetic acid. Potassium nitrite cannot be used because the reaction of this nitrite with glacial CH_3COOH is too vigorous. Where caprylic alcohol is used to overcome foaming a new blank must be run. The speed of shaking should also be maintained as nearly constant as possible. The time required to rid the reaction vessel of air may be materially lessened by warming the nitrite to 30° C. and shaking by hand during this preliminary process. The motor, however, adjusted to a suitable and definitely maintained speed, should always be used for the reaction shaking. To obtain reliable results the above precautions and the following, as advised by Dr. D. W. Wilson, Department Physiological Chemistry, Johns Hopkins Medical School, should be carefully observed. The stopcocks, especially the one just above the gas burette, should be kept well greased with the vaseline-paraffin-rubber preparation recommended by van Slyke; this should be done after every 3 or 4 determinations. After 200–300 determinations the stopcocks should be ground gently with powdered emery and oil followed by talcum in water. The method is not very satisfactory for solutions containing peptones or proteins; accordingly the results given for "peptid" N of the two peptone media are to be more dependable than those for "total amino."

In the procedure for determining "peptid" N a 50-ml. portion of the medium was hydrolyzed 3 to 5 hours with 20 per cent HCl,

the "humin" N filtered off, and the filtrate and washings then neutralized with saturated NaOH solution and made to volume (100 ml.). A portion of this solution was made distinctly alkaline to phenolphthalein, aerated to rid it of amide and ammonium N, and the "peptid" N estimated by the van Slyke "micro" method, as in the amino N determinations. The results for amino N theoretically should be subtracted from the results obtained here to give the N that was bound in the peptid linkings and freed by hydrolysis, but this was not done owing to the questionable accuracy of the former. Hydrolysis of culture fluid from the 3 mineral N media (numbers 3, 4, and 5) was not found to increase the NH_3.N content, so it was reasoned that higher peptids, or proteins, are not excretion products of the 3 fungi cultured on these media.

From the NH_3 obtained by the aeration of the hydrolyzed material was subtracted that of the ammonia determination and the result called "amide" nitrogen.

Data and Discussion

A preliminary experiment was carried out to obtain an indication of the capacity or inability of *Sphaeropsis malorum* and *Diplodia natalensis* to utilize elementary N_2. These fungi are sphaeropsidaceous forms, as is the *Phoma Betae* which was shown to have the capacity for fixing free N_2. The Ascomycete, *Nectria Ipomoeae,* was also used in this experiment. The technique described by Duggar and Davis ('16) was followed exactly except that 1-liter, round-bottom Jena flasks were used as the culture, digestion, and distillation flasks. The cultures were incubated in darkness at a temperature of 28° C. Duggar's solution for fungi was used as the culture medium, the carbon source being common cane sugar instead of glucose, and the N source 2 different concentrations of peptone solution. The results here given show the total incapacity of these 3 forms to fix the N_2 of the air under the conditions of the experiment.

TABLE III

NITROGEN FIXATION

Organism	Days incubation	Mgm. N in culture	Mgm. N in check
Nectria Ipomoeae	14	34.82	
	14	35.11	
	14	34.77	34.82
	14	35.39	34.76
	14	35.05	
Nectria Ipomoeae	14	18.09	17.98
Nectria Ipomoeae	29	34.94	
	29	34.82	34.82
Sphaeropsis malorum	14	34.26	
	14	35.56	34.71
	14	34.94	34.71
Sphaeropsis malorum	14	17.98	
	14	18.09	17.98
	14	18.38	18.03
Sphaeropsis malorum	29	34.65	
	29	34.71	34.71
Diplodia natalensis	14	36.19	
	14	35.51	35.79
Diplodia natalensis	14	18.72	
	14	18.78	18.09
	14	19.00	17.93
Diplodia natalensis	29	35.34	
	29	35.79	35.79

The analytical data for the work with *Aspergillus niger*, *Sphaeropsis malorum*, and *Diplodia natalensis* are given in the following tabulations and graphs. The heavy vertical lines of the graphs represent the days on which determinations were made. The [$\overset{+}{\mathrm{H}}$] of the "peptone plus dextrose" medium is seen to increase during the first 3 to 5 days in cultures of all 3 fungi; a comparison of the curves for dry weight and [$\overset{+}{\mathrm{H}}$] shows that the rise in [$\overset{+}{\mathrm{H}}$] is proportional to the rapidity and amount of growth of the fungi. For the very fast-growing *Aspergillus* the maxima of growth of the organism and [$\overset{+}{\mathrm{H}}$] of the medium are coincident in time; whereas for the other 2 fungi there is a distinct lag of 2 to 3 days in the growth maximum. That both these factors are intimately related to the carbohydrate consumption is

evident. With the disappearance of the dextrose in *Aspergillus* cultures a decrease in the weight of the fungus and in $[\overset{+}{H}]$ immediately begins. For the other 2 fungi the maximum dry

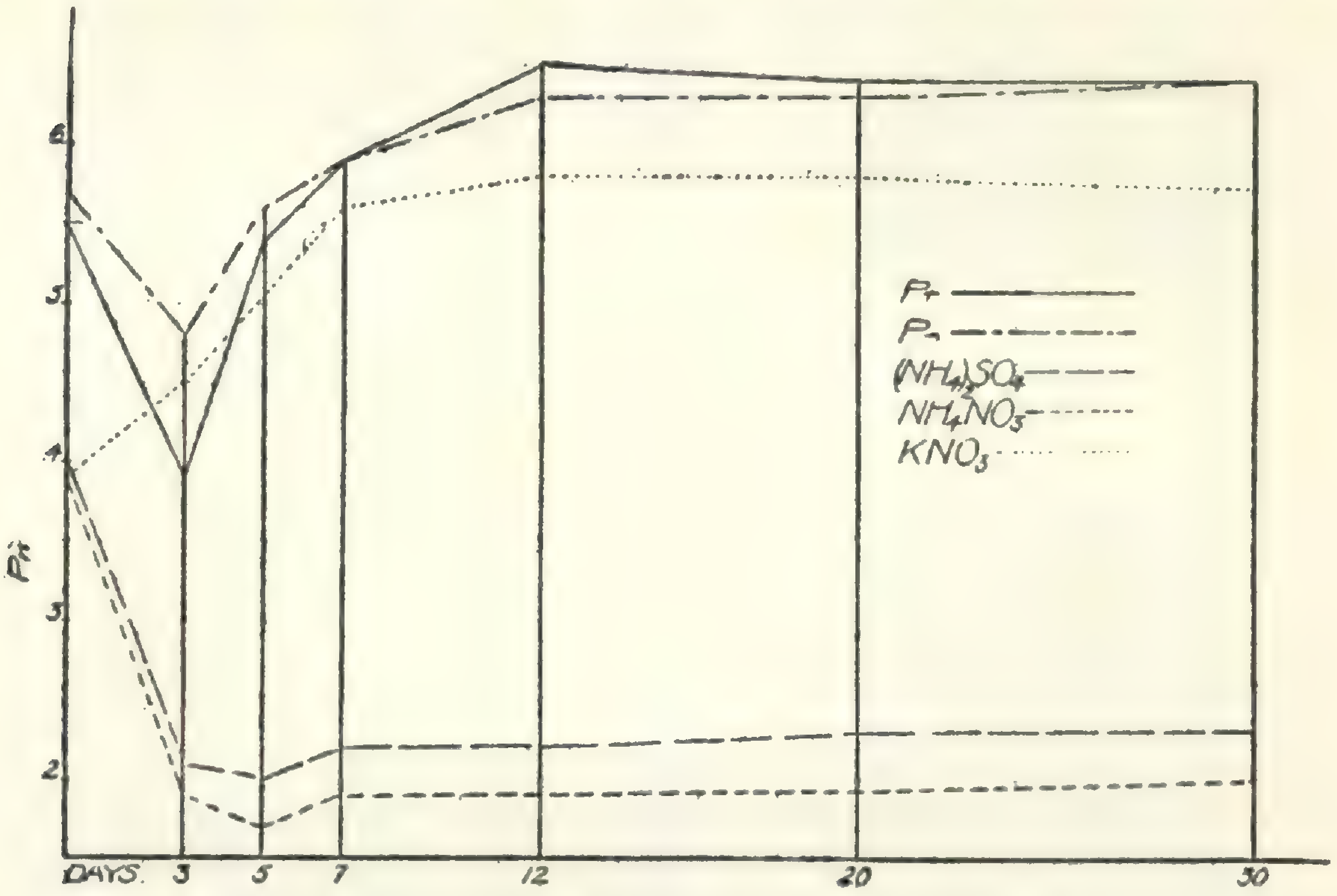


Fig. 1. H-ion change of media. *Aspergillus niger*, first series.

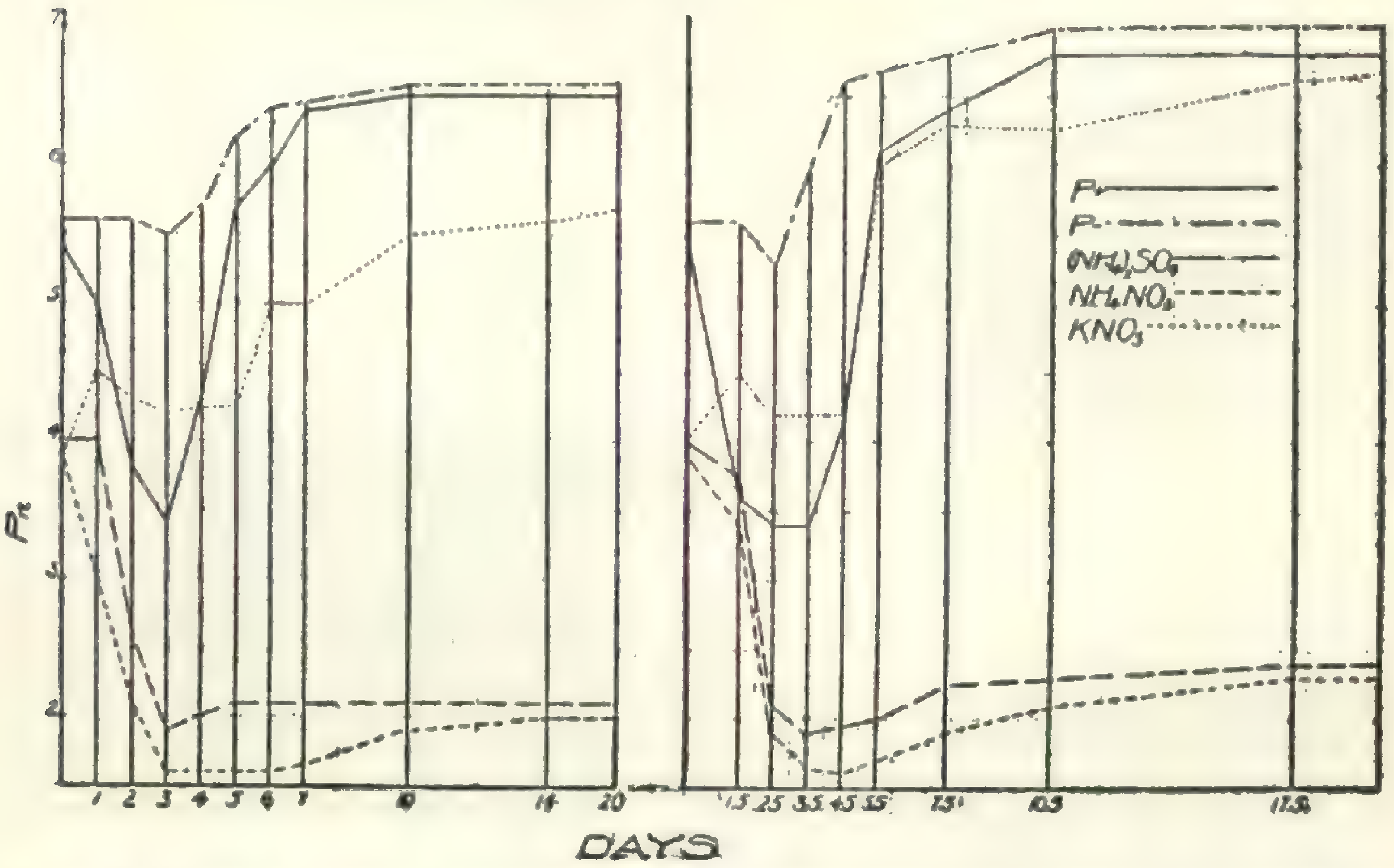


Fig. 2. H-ion change of media. *Aspergillus niger*, second (left) and third series.

weight and the point of disappearance of the sugar are synchronous. The organic acids produced in the decomposition of the glucose are probably responsible for the rapid elevation of H-ion concentration. The carbohydrate having been entirely transformed, the organic acids are rapidly built up into the substance of the fungus and consequently there is a rapid decrease in $[\overset{+}{H}]$, as indicated by the rapidly ascending curve for the $[\overset{+}{H}]$ exponent, P_H. This would explain the growth lag evidenced by the *Sphaeropsis* and *Diplodia*. This would probably have been found to be the case also with the faster-growing *Aspergillus*, had determinations been made at intervals of 5 or 6 hours rather than days.

TABLE IV

CHANGES IN H-ION CONCENTRATION, DEXTROSE, TOTAL N, AND NITRITE N OF THE MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

ASPERGILLUS NIGER—FIRST SERIES

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Dry wt. Mat No. 1	Dry wt. Mat No. 2	Dry wt. Mat No. 3	Average dry wt. mats	Total N	NO_2. N	Dextrose
	0	5	5.5					228.4	0	2477
Peptone	3	5	3.9	1837	1810	1713	1787	120.0	.0012	Trace
plus	5	5	5.4	1321	1290	1336	1316	125.2	.0010	0
dextrose	7	5	5.9	1172	1192	1030	1132	149.5	0	
	12	5	6.5	1116	1078	1129	1108	154.7	.0125	
No. 1	20	5	6.4	1116	1120	1144	1127	139.1	.0073	
	30	5	6.4	1069	1070	1045	1061	146.1	.0025	
Peptone	0	5	5.7					214.5	0	0
minus	3	5	4.8	222	271	232	242	189.2	.0005	
dextrose	5	5	5.6	338	263	312	304	208.6	.0007	
	7	5	5.9	294	258	258	273	208.6	.0087	
No. 2	12	5	6.3	249	237	238	241	187.8	.0062	
	20	5	6.3	250	252	227	243	196.5	0	
	30	5	6.4	224	255	177	219	201.7	.0020	
	0	5	4.0					284.0	0	2477
	3	5	2.1	1009	981	923	971	246.9	.0005	365
$(NH_4)_2SO_4$	5	5	2.0	1060	982	1149	1063	236.4	.0002	0
	7	5	2.2	907	830	937	891	239.9	.0001	
No. 3	12	5	2.2	703	682	740	708	246.9	0	
	20	5	2.3	856	634	682	724	245.1	0	
	30	5	2.3	644	677	742	688	255.6	0	

TABLE IV (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Dry wt. Mat No. 1	Dry wt. Mat No. 2	Dry wt. Mat No. 3	Average dry wt. mats	Total N	NO_2. N	Dextrose
NH_4NO_3 No. 4	0	5	3.9					280.0	.0010	2477
	3	5	1.9	1161	1116	1199	1159	219.1	.0005	Trace
	5	5	1.7	1017	1044	1044	1035	205.2	.0005	0
	7	5	1.9	927	885	913	908	227.8	0	
	12	5	1.9	818	864	865	849	246.9	0	
	20	5	1.9	775	765	877	806	239.9	0	
	30	5	2.0	766	722	747	745	248.0	0	
KNO_3 No. 5	0	5	3.9					149.5	.0005	2477
	3	5	4.5	821	993	888	901	97.4	.0025	787
	5	5	5.5	1031	1000	1189	1073	90.4	.0067	0
	7	5	5.6	791	820	889	830	95.6	.0162	
	12	5	5.8	740	778	732	750	90.4	.0085	
	20	5	5.8	786	702	737	742	104.3	.0010	
	30	5	5.7	804	633	738	725	111.3	0	

TABLE V

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

ASPERGILLUS NIGER—SECOND SERIES

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	NH_4.+NH_3.N	Total N	NO_3.N	NH_2.N	$CONH_2$.N	CONH.N
Peptone plus dextrose No. 1	0	5	5.4	0	2255	1.38	176.7	0	27.23	4.14	76.51
	1	1	5.0	113	2212						
	2	1	3.8	615	1235						
	3	5	3.4	1445	47	1.38	86.94		7.56	4.14	46.7
	4	1	4.3	1279	18						
	5	5	5.7	1032	18	30.36	102.8		10.1	2.76	37.64
	6	1	6.0	1110							
	7	5	6.4	1034		33.81	91.77		10.38	3.45	30.44
	10	5	6.5	1004		46.92	113.2		12.73	1.1	37.23
	14	5	6.5	985		48.3	108.3		11.72	0	32.72
	20	5	6.5	946		51.06	106.9		12.88	12.4	28.51
Peptone minus dextrose No. 2	0	5	5.6	0	0	2.76	176.7	0	31.6	2.76	93.58
	1	1	5.6	60							
	2	1	5.6	225							
	3	5	5.5	157		35.88	153.2		11.6	13.8	54.8
	4	1	5.7	215							
	5	5	6.2	168		64.86	160.1		16.05	6.9	49.36
	6	1	6.4	233							
	7	5	6.4	199		68.31	152.5		11.21	2.07	44.83
	10	5	6.6—	187		77.28	162.8		16.1	0	43.25
	14	5	6.6—	178		80.04	162.1		13.08	2.76	41.71
	20	5	6.6	156		78.66	160.1		14.25	13.8	49.07

TABLE V (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	$NH_4+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	4.0	0	2255	274.0	277.7	0	0	0	0
	1	1	4.0	85	2244						
	2	1	2.6	387	1525						
$(NH_4)_2SO_4$	3	5	1.9	1115	88	205.6	211.2		0		
	4	1	2.0	1045	3						
No. 3	5	5	2.1	822		229.1	227.0		.27		
	6	1	2.1	882							
	7	5	2.1	866		193.2	197.3		.97		
	10	5	2.3	816		209.7	222.2		1.52		
	14	5	2.3	761		219.4	231.2		4.9		
	20	5	2.3	754		229.7	233.2		1.23		
	0	5	3.9	0	2255	143.1	285.7	142.6	1.1	0	0
	1	1	4.0	69	2225						
	2	1	2.1	425	1674						
NH_4NO_3	3	5	1.6	866	185	96.6	221.5	124.9	0		
	4	1	1.6	1039	7						
No. 4	5	5	1.6	918		95.2	233.6	138.4	.27		
	6	1	1.6	1006							
	7	5	1.7—	899		87.3	224.3	137.0	7.26		
	10	5	1.9+	733		116.3	249.8	133.5	2.21		
	14	5	2.0+	697		118.7	260.8	142.1	1.36		
	20	5	2.0+	639		117.3	258.7	141.4	1.1		
	0	5	3.9	0	2255	1.38	142.8	141.4	0	0	0
	1	1	4.5	98	2243						
	2	1	4.3	345	1770						
KNO_3	3	5	4.2	413	1082	1.38	104.9	103.5	0		
	4	1	4.0	756	581						
No. 5	5	5	4.0	833	72	1.38	95.2	93.8	.54		
	6	1	5.0	786	5						
	7	5	5.0	792	4	2.07	95.2	93.1	.55		
	10	5	5.5	657		4.14	110.4	106.3	.83		
	14	5	5.6	637		2.76	112.5	109.7	2.04		
	20	5	5.7	667		4.83	107.7	102.8	.55		

TABLE VI

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

ASPERGILLUS NIGER—THIRD SERIES

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. mats (mgm.)	Dextrose	NH_4+NH_3.N	Total N	NO_3.N	NH_2.N	$CONH_2$.N	CONH.N
	0	5	5.4	0	2540	1.38	176.7	0	27.52	4.14	76.51
	1½	1	3.6	186	1920						
Peptone	2½	1	3.4	738	982						
plus	3½	5	3.4	1512	55.6	4.14	93.84		10.8	0	43.1
dextrose	4½	1	4.1	1549	5.2						
	5½	5	6.1	1235	.0	22.1	82.8		8.7	1.38	34.9
No. 1	7½	5	6.4	1168		26.2	82.8		7.01	4.14	26.6
	10½	5	6.8	928		37.2	96.6		9.24	2.82	24.6
	17½	5	6.8	852		42.1	93.32		8.9	2.74	26.9
	27	5	6.8	865							
	0	5	5.6	0	0	2.7	176.7	0	32.7	2.76	93.6
	1½	1	5.6	73							
Peptone	2½	1	5.3	225							
minus	3½	5	6.0	212		37.3	152.5		14.8	0	61.3
dextrose	4½	1	6.6	192							
	5½	5	6.7	218		55.2	152.0		16.2	5.52	59.9
No. 2	7½	5	6.8	214		56.6	151.8		11.2	2.76	43.6
	10½	5	7.0	183		63.5	151.8		10.6	1.38	36.0
	17½	5	7.0	198		69.0	153.2		14.1	0	39.8
	27	5	7.0	199							
	0	5	4.0	0	2540	274.	277.7		0	0	0
	1½	1	3.5	160	2280						
$(NH_4)_2SO_4$	2½	1	2.1	580	1500						
	3½	5	1.9	1126	213	212.5	216.6		2.27		
No. 3	4½	1	2.0—	1144	0						
	5½	5	2.0	1032		213.9	234.6		2.8		
	7½	5	2.3—	767		200.1	205.6		1.68		
	10½	5	2.3	744		204.2	209.7		1.96		
	17½	5	2.4	684		200.1	204.2		2.25		
	27	5	2.4	646							
	0	5	3.9	0	2540	142.2	285.6	139.4	0	0	0
	1½	1	3.4	86	2310						
NH_4NO_3	2½	1	1.9	510	1533						
	3½	5	1.7—	897	86.4	96.6	220.8	123.9	.43		
No. 4	4½	1	1.6	965	2.9						
	5½	5	1.7	919		96.6	226.3	125.6	1.96		
	7½	5	1.9	796		103.5	234.6	128.3	1.68		
	10½	5	2.1	680		113.1	247.	128.3	.84		
	17½	5	2.3	616		113.1	245.7	127.0	1.41		
	27	5	2.3	616							

TABLE VI (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Average dry wt. mats	Dextrose	$NH_4+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	3.9	0	2540	1.3	142.8	138.0	0	0	0
	1½	1	4.5	90	2390	trace					
KNO_3	2½	1	4.2	410	1705						
	3½	5	4.2	607	1507		107.6	102.1			
No. 5	4½	1	4.2	736	242						
	5½	5	6.0	868	18	1.38	95.2	86.94	1.82		
	7½	5	6.3	649		5.52	104.9	96.6	2.8		
	10½	5	6.2+	602		4.14	115.9	97.98	.56		
	17½	5	6.6	612		9.66	109.2	95.22	1.13		
	27	5	6.8	525							

TABLE VII

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

SPHAEROPSIS MALORUM

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	$NH_4.+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	5.5	0	2452	1.5	154.8	0	33.2	7.6	90.1
	2	5	5.4	14	2414	1.6	150.2				
Peptone	3	5	5.0	124	2048	1.6	143.4				
plus	4	5	4.5	382	1452	1.6	129.7				
dextrose	5	1	4.0	884	415		96.7				
	6	5	4.4	1078	68	1.8	72.8				
No. 1	7	2	5.2	1293	16	3.4	68.3		16.7	3.4	39.2
	12	5	7.8	1114		31.8	79.7				
	16	2	8.1	914		44.4	88.7		13.4	1.12	25.66
	35	1	7.8	594		48.9	109.3		18.7		
	0	5	5.7	0	0	1.5	154.8	0	36.8	2.14	95.7
	2	5	5.8	4		1.8	153.6				
Peptone	3	5	5.9	15		3.18	154.8				
minus	4	5	6.2	52		7.96	152.5				
dextrose	6	5	6.6	159		18.2	136.6				
	7	2	6.9	192		23.9	129.7		21.1	5.7	61.12
No. 2	12	5	7.6	215		37.55	132.0				
	16	5	8.1	185		44.3	132.0		27.1	.7	48.75
	35	1	7.8	115		45.5	130.9		23.2		

TABLE VII (*Continued*)

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	$NH_4+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	CONH.N
	0	5	4.2	0	2452	245.8	247.0	0	1.4	0	0
	2	1	4.1	2	2439	245.8	247.				
	3	1	4.0	12	2428	245.3	247.				
$(NH_4)_2SO_4$	6	5	3.6	39	2247	243.9	245.3				
	8	5	3.3	98	1671	239.4	242.4		2.25		
No. 3	9	2	2.8	146	1210						
	12	5	2.8	160	886	232.1	235.6				
	15	1	2.7								
	17	2	2.7	193	680	228.7	233.9		3.9		
	0	5	4.0	0	2452	124.4	250.3	125.9	.56	0	0
	2	5	4.0	3	2452	124.4	246.9	122.5			
	3	5	4.0—	3	2452	124.4	246.9	122.5			
NH_4NO_3	6	5	3.8	67	2274	122.9	246.9	122.5			
	8	5	3.8+	207	1651	118.8	237.8	119.0	.7		
No. 4	9	2	3.8+	363	1090						
	10	1	4.4	365	836						
	12	5	5.0	374	270	116.0	223.0	107.0			
	15	1	5.8	655	16						
	16	5	5.8	574		112.7	217.3	104.6	.57		
	0	5	4.0	0	2452	0	121.8	121.8	0	0	0
	2	5	4.0	5	2450	0	119.5	119.5			
	3	5	4.0	4	2450	0	119.5	119.5			
KNO_3	6	5	4.9	95	2112	0	119.5	119.5			
	8	5	5.7	377	1419	0	102.4	102.4	.14		
No. 5	9	2	5.8	523	779	0					
	10	1	5.8	733	176	0					
	12	5	6.0	761	69	0	88.7	88.7			
	16	2	6.6	715	12	0	88.7	88.7	.14		
	35	2	8.1	376		5.7	102.4	96.7	.14		

TABLE VIII

CHANGES IN H-ION CONCENTRATION, DEXTROSE, AND NITROGEN CONSTITUENTS OF MEDIA, AND IN DRY WEIGHT OF FUNGOUS MAT

DIPLODIA NATALENSIS

All weights expressed in mgm. per 50 ml. of media

Medium	Days incubation	Number of cultures	P_H	Average dry wt. fungous mat	Dextrose	$NH_4+NH_3.N$	Total N	$NO_3.N$	$NH_2.N$	$CONH_2.N$	$CONH.N$
Peptone plus dextrose No. 1	0	5	5.4	0	2367	0	154.77	0	33.22	9.1	90.1
	2	1	5.0	85	2276						
	3	5	4.7	279	1842	0	128.6				
	4	2	4.6	668	789	0					
	5	5	5.0	821	194	4.5	94.45				
	6	2	6.5	925	73	17.1					
	8	5	6.9	849	7 da. 63	22.8	91.04		19.7	4.55	46.23
	12	5	8.1	703		40.97	100.14				
	18	5	8.2	615		42.11	97.9		13.54	3.41	35.17
	38	2	8.2	597		42.11	91.04		11.15		28.02
Peptone minus dextrose No. 2	0	5	5.7	0		0	154.77	0	36.87	2.45	95.7
	3	5	5.8	15		3.	153.6				
	4	2	6.3	86							
	5	5	6.6	133		25.	144.5				
	6	2	7.0	185		25.					
	8	5	7.5	248		35.3	132.0		23.74	3.4	56.72
	11	1	8.3	259							
	12	5	8.4	251		39.8	128.6				
	18	5	8.5	223		44.38	119.5		19.12	5.69	42.22
	38	2	8.4	203		43.24	116.1		17.44		31.45
$(NH_4)_2SO_4$ No. 3	0	5	4.2	0	2367	245.8	246.9	0	1.4	0	0
	3	5	3.9	28	2360	243.5	243.5				
	4	2	3.4	78	2180						
	5	5	2.8	124	1606	233.3	236.7				
	6	2	2.7	203	1006	225.3					
	8	5	2.6	186	718	220.7	234.4		4.5		
	10	1	2.6	193	511						
	12	5	2.6	217	509	223.	232.1				
	18	5	2.6	232	428	220.	229.8		6.0		
	38	2	2.6	355	41	208.3	220.8		6.0		
NH_4NO_3 No. 4	0	5	4.0	0	2367	124.1	250.4	126.3	.56	0	0
	3	5	3.9	23	2360	124.	245.8	121.8			
	4	2	3.8	45	2297						
	5	5	3.4	133	1845	119.5	240.1	120.6			
	6	2	3.2	187	1492	118.35					
	8	5	3.3	387	67	104.7	219.6	114.9	1.7		
	9	1	3.6	415	21						
	12	5	2.9	474		102.4	213.9	111.5			
	18	5	2.7	368		101.3	221.9	120.6	3.9		
	38	2	2.7	391		101.3	216.2	114.9	4.0		

TABLE VIII (*Continued*)

Medium	Days incubation	Number of cultures	PH	Average dry wt. fungous mat (mgm.)	Dextrose	NH_4.+NH_3.N	Total N	NO_3.N	NH_2.N	$CONH_2$.N	CONH.N
KNO_3 No. 5	0	5	4.0	0	2367	0	121.8	121.8	0	0	0
	3	5	4.1	30	2295	0	111.5	111.5			
	4	2	4.8	53	2230						
	5	5	5.5	271	1654	0	104.7	104.7			
	6	2	5.7	326	1554	0					
	8	5	5.8	632	413	0	87.63	87.63	1.13		
	9	1	7.0	759	28						
	12	5	7.1	613		2.2	87.6	85.4			
	18	5	7.8	485		7.97	96.73	88.76	1.26		
	38	1	7.8	422		7.97	89.9	81.93	1.28		

At this point in the growth of the fungi, since the carbohydrate carbon supply has been exhausted, the organisms must draw upon the peptone and probably upon their own substance for both C and N to continue their metabolic processes, especially

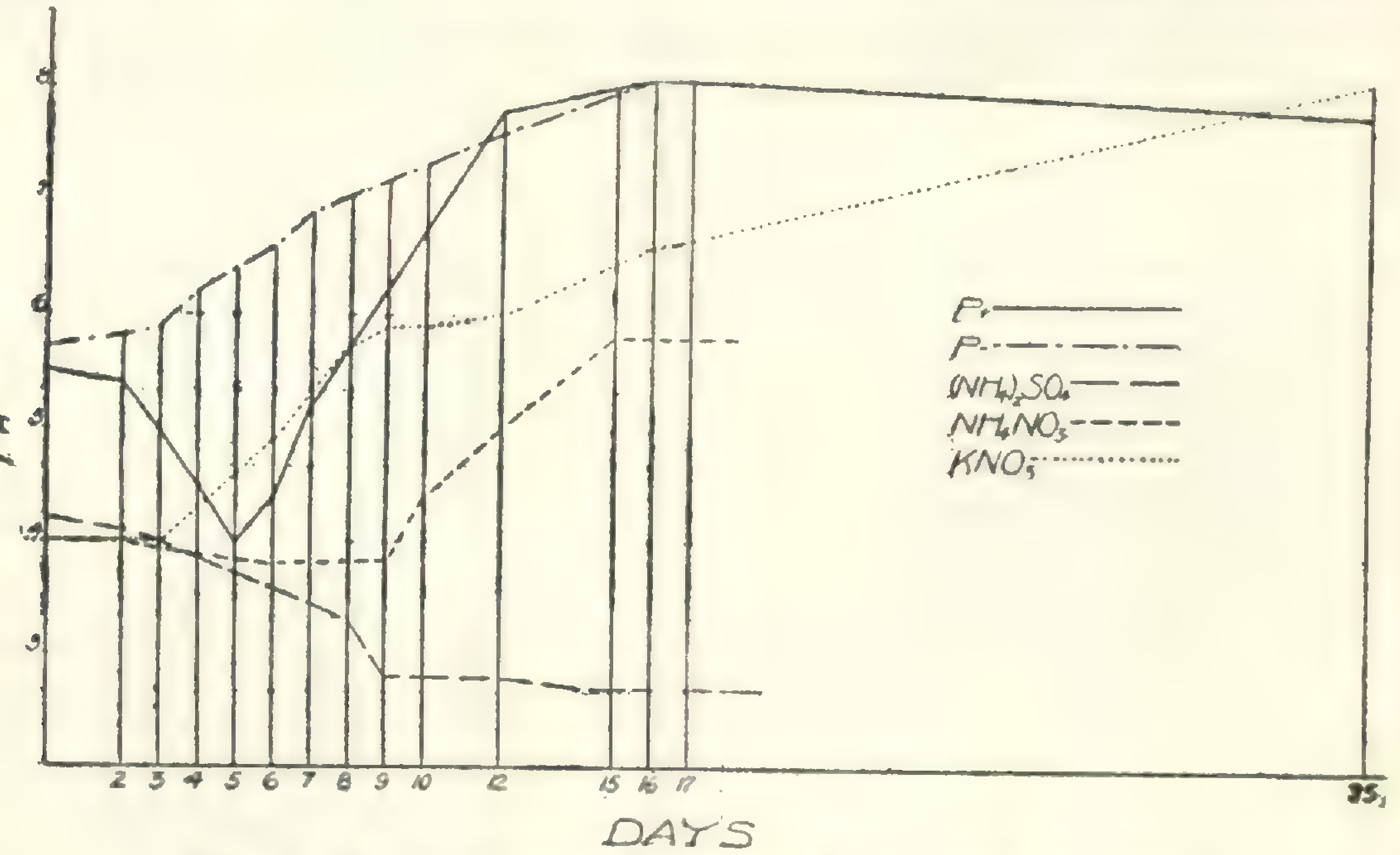


Fig. 3. H-ion change of media. *Sphaeropsis malorum*.

respiration. With the disappearance of the sugar there is a relatively rapid increase in the NH_4.N of the medium. At least 2 factors may enter into an explanation of this. The proportion of N to the non-nitrogenous complex of the peptone is greater

than is required in respiration, protein synthesis, and other life processes; accordingly the excess N appears as NH_3. In the case of *Aspergillus niger* this rapid excretion of NH_3 in the "peptone minus dextrose" medium comes about 2 days before that in the "peptone plus dextrose" medium, and respectively about 3 and 5 days earlier for cultures of *Diplodia* and *Sphaeropsis*, showing the protective action of the sugar. Decrease in weight of the fungus indicates autolysis and the consequent diffusion of autolytic products into the medium. Ammonia is very likely one of the products of this autolysis. The results for

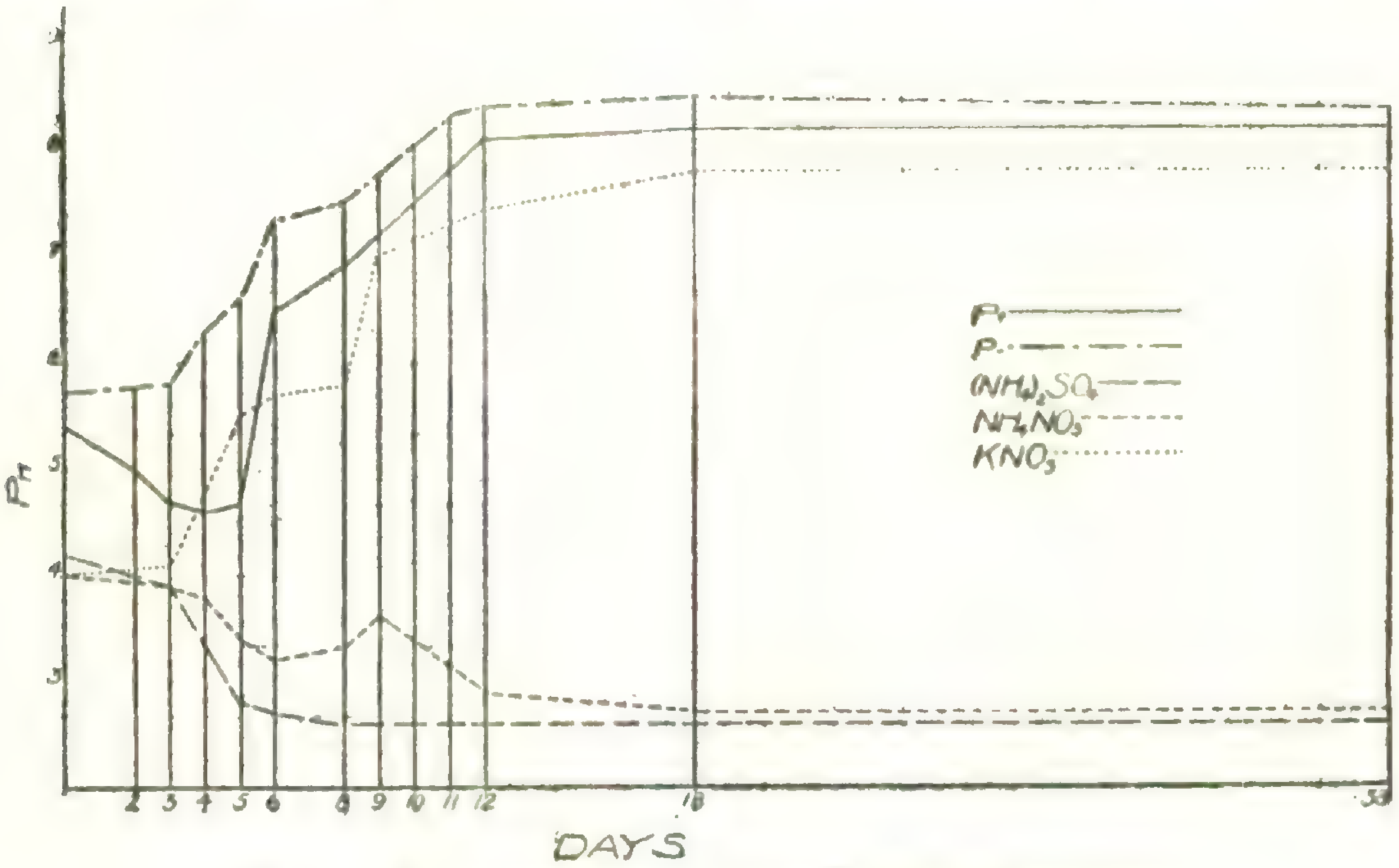


Fig. 4. H-ion change of media. *Diplodia natalensis.*

total N are also strikingly suggestive in this regard. With the decrease in weight of the fungi the N content of the media increases above its minimum, indicating that the autolytic products are in part nitrogenous. The results for amino, amide, and peptid N are not suggestive in this regard; in fact the peptid N of the medium in general decreased in quantity during the incubation of all 3 fungi. There is, however, a slight increase in the peptid N content of the medium of the *Aspergillus* cultures at the end of the incubation. These results indicate that NH_3.N is the chief nitrogenous product of autolysis. The quantity of NH_3 excreted reaches nearly a third of the total N of the medium in 18 to 20

days. The determinations, moreover, corroborate those of Dox and Maynard ('12 and '13).

While it is not known that NH_3 is an end product of protein metabolism, it is certain that it is directly related to autolytic processes; and in cultures where peptone or protein is the sole source of C and N, NH_3 is produced in excess by the breaking down of large numbers of molecules and the use of the non-N-containing parts for energy. In the experiment on N fixation

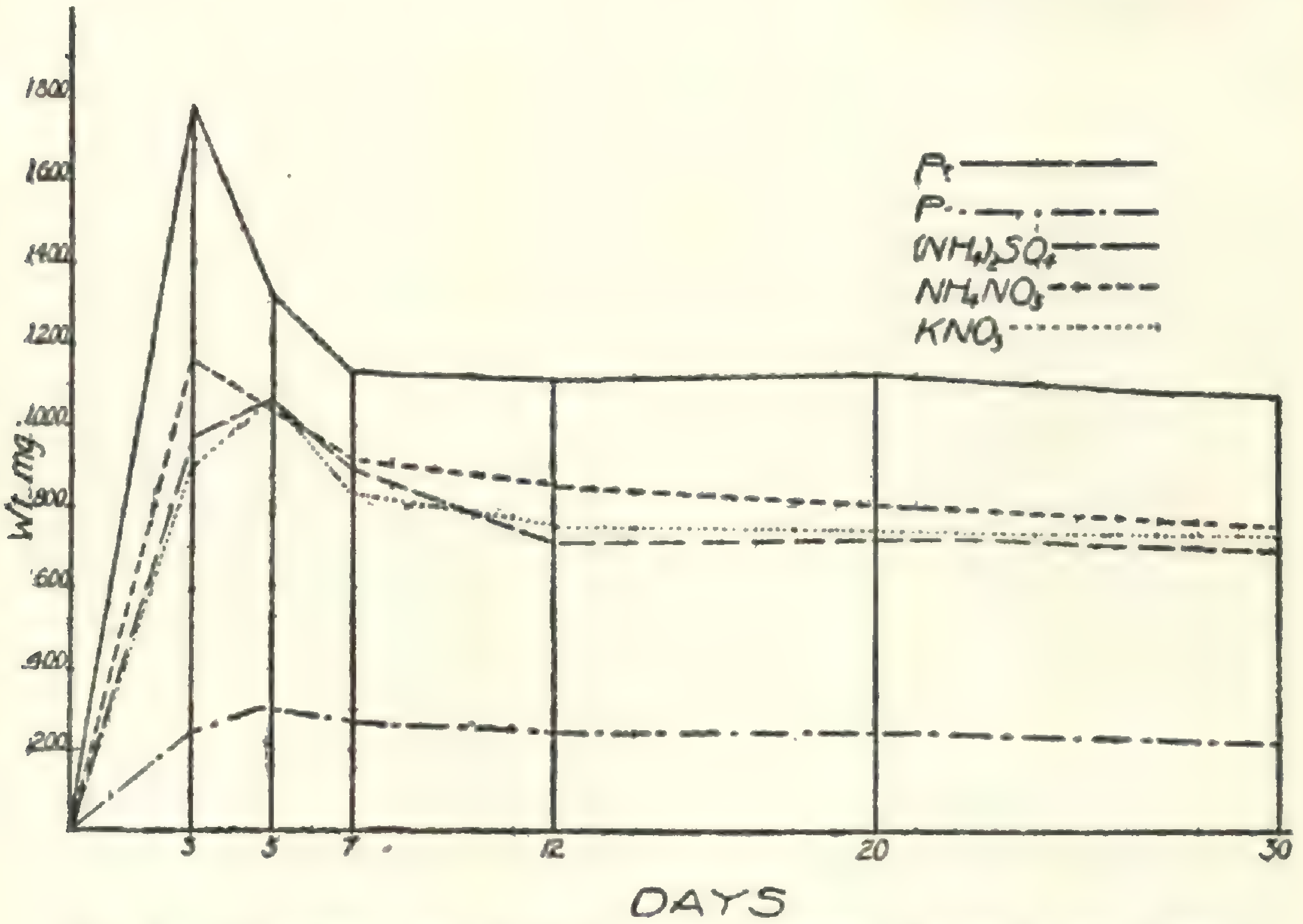


Fig. 5. Dry weights of fungous mats. *Aspergillus niger*, first series.

reported at the beginning of this discussion, it is significant that the N content of the cultures remained constant for 29 days. The initial N content of the media was very small, and any ammonia formed from the peptone in the presence of the abundant sugar was immediately reassimilated and retained. There was consequently no loss of N during 29 days of incubation. On the other hand, in the cultures of *Sphaeropsis* and *Diplodia* where the peptone content was large and the sugar disappeared in 7 to 8 days, there was a distinct loss of nitrogen when the cultures became alkaline. The strong odor indicated that the loss was due to the evolution of free NH_3. At the end of 15 days a culture

of *Sphaeropsis malorum* on the P medium was found, by Kjeldahlization of the mat and solution collectively, to have lost 6.77 mgm. of N; and one on P medium, 3.42 mgm. Similarly a 20-day P+ culture of *Diplodia natalensis* lost 7.97 mgm. N, and a 20-day P— culture, 9.106 mgm. On the 3 media in which inorganic N served as the N source none of the cultures decreased in total N content. The continued acidity of the $(NH_4)_2SO_4$ and NH_4NO_3 media prevents the evolution of NH_3, while the small amount of NH_3 produced in alkaline KNO_3 cultures is

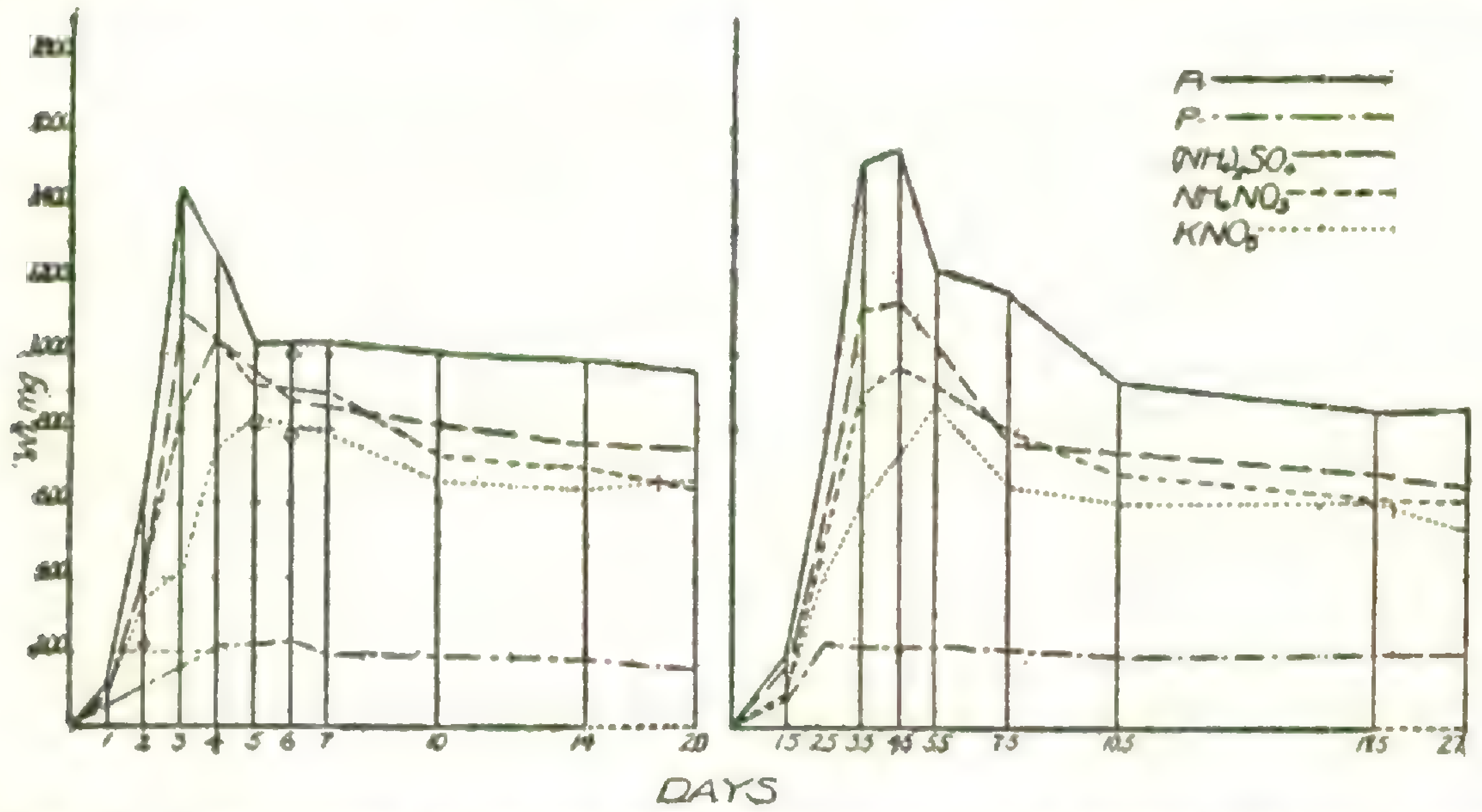


Fig. 6. Dry weights of fungous mats. *Aspergillus niger*, second and third series.

precipitated as $MgNH_4PO_4.6H_2O$ or easily held in solution as NH_4OH. This strengthens the probability that the NH_3 evolved under the alkaline conditions of the P+ and P— media is responsible for the N loss in these cultures.

Waksman ('18) did not make sugar- and dry-weight determinations and consequently could not legitimately connect the appearance of ammonia with autolysis and the disappearance of sugar. The sugar of his cultures had probably disappeared at the end of 3 to 5 days and consequently the obedience of the accumulation of NH_3 to the law of autocatalysis could not have been due to the presence of the carbohydrate, as he claimed. He is probably correct in assuming that a definite quantity of NH_3 may always be produced from protein materials, as a waste product, independently of the presence of available carbohydrate.

My results show that this ammonia is, as Waksman assumes, "reassimilated in the presence of available carbohydrate by the organisms that are able to utilize it readily as a source of N." In the case of *Sphaeropsis* and *Aspergillus* "the production of NH_3 was entirely prevented by the presence of the sugar." The relation also holds with *Diplodia* but in cultures of this fungus an appreciable quantity of NH_3 (17.1 mgm. per culture) appeared when 73 mgm. of glucose were still present. This may have

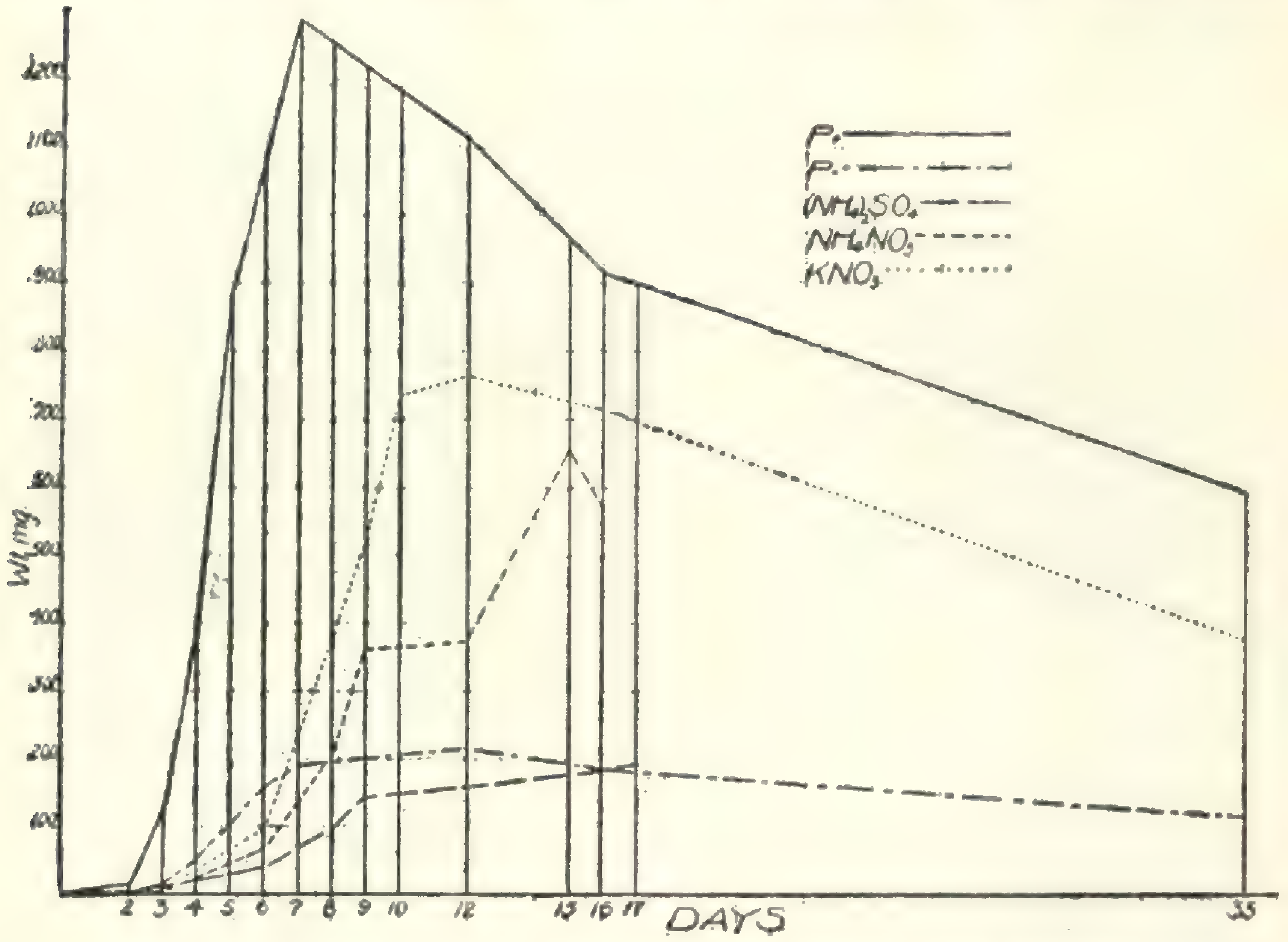


Fig. 7. Dry weights of fungous mats. *Sphaeropsis malorum.*

been due to the fact that the aerial, folded growth of the fungus minimized contact with the medium and thereby lessened absorption from it. In the absence of the dextrose, the strongly proteolytic organisms use the peptone as a source of C and leave much of the N to diffuse into the medium as NH_3.

As the alkalinity advanced beyond P_H 7.0 crystals formed in all the peptone media for all 3 fungi and in the KNO_3 medium of *Sphaeropsis* and *Diplodia* cultures. In the peptone cultures a strong odor of NH_3 was apparent, and crystals of $MgNH_4PO_4 \cdot 6H_2O$ were precipitated; in the KNO_3 medium with the *Diplodia*

and *Sphaeropsis* the precipitate was largely $Mg_3(PO_4)_2 4H_2O$, as the NH_3 content of these cultures does not reach an appreciable quantity until late in the incubation, when most of the Mg and PO_4 ions have been precipitated. The appearance of the characteristic crystals of the salts may be considered as a rough indication of the beginning of an alkaline reaction which, for the most part, results from predominance of autolytic processes. The reduction in active acidity necessary to their formation was determined experimentally by adding NH_4OH or KOH to the media; the

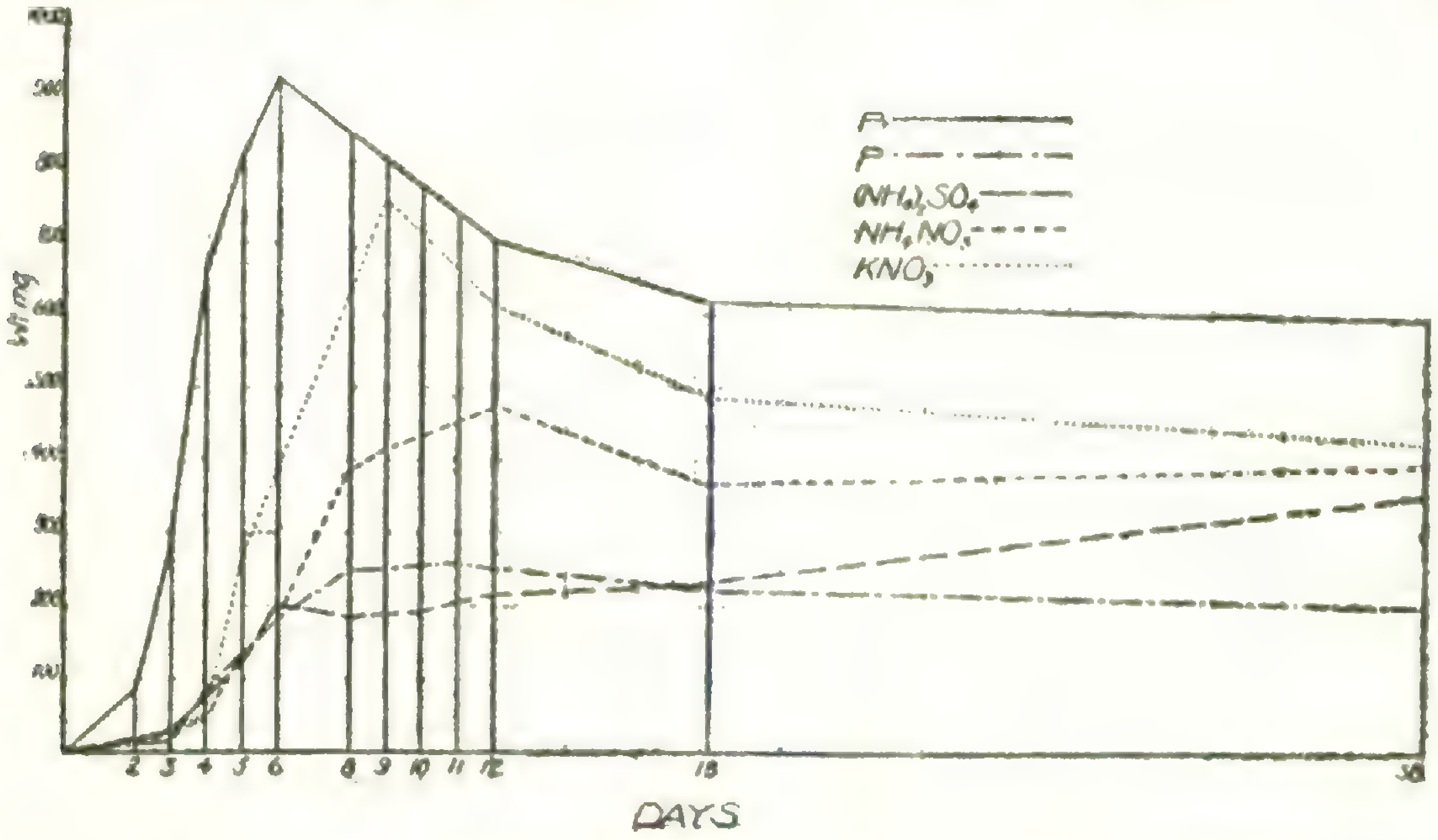


Fig. 8. Dry weights of fungous mats. *Diplodia natalensis.*

crystals were centrifuged out and identified by microscopic comparison with known salts and by analyzing quantitatively for N content and qualitatively for the ions present. Crystals appeared at P_H 6.1 in the presence of NH_3; that is, when NH_4OH was added to the media, or KOH to media 3 and 4 which contained NH_4 salts, these were identified as $MgNH_4PO_4.6H_2O$. When KOH was added to media 1, 2, and 5, crystals were not formed until the exponent P_H 7.1–7.2 was reached; these were $Mg_3(PO_4)_2.4H_2O$.

The causes of the slight fall in the P_H curve of the "peptone minus" cultures of *Aspergillus niger* during the first 3 days of incubation is problematic. Possibly during rapid formation of protoplasm of the young mycelial threads the NH_2 group of the

amphoteric amino acids is slightly more utilized than the non-nitrogenous part, leaving some carboxyl groups in excess. It appears probable that this differential utilization by the extremely fast-growing young germ tubes from the many spores of the inoculum produces a brief state of hyperacidity before autolysis, respiration, and other processes which make for alkalinity have come into full play. This temporary increase in acidity was not produced by *Sphaeropsis* and *Diplodia* in the P— medium; with these fungi the inoculum was a relatively large quantity of

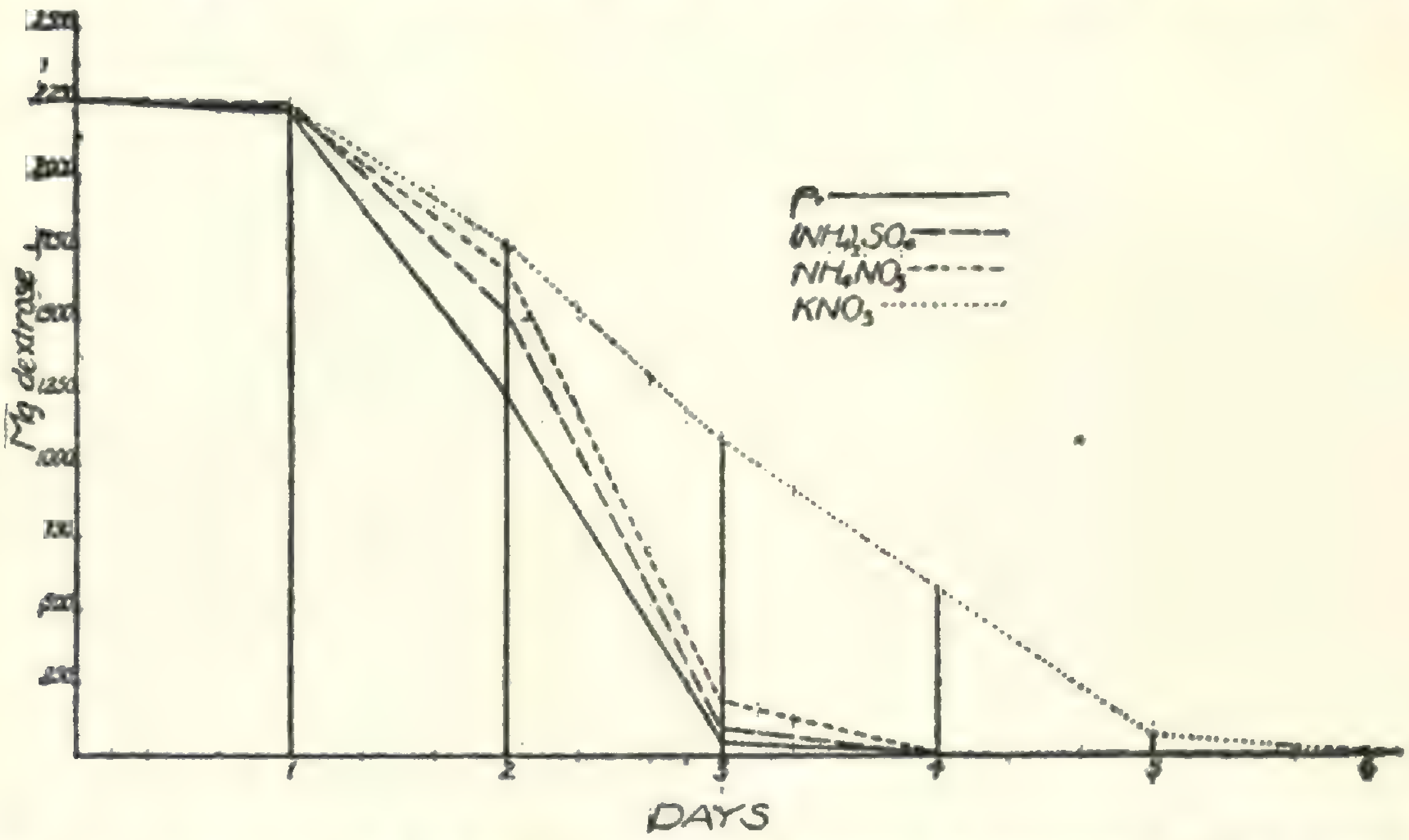


Fig. 9. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media *Aspergillus niger*, second series.

mature mycelium in which respiration and autolytic processes were already predominant. The amount of autolysis, as indicated by decrease in dry weight from the maximum, is proportional to the weight the fungous mat attains.

The fact that after the period of rapid development of the fungus, the amount of NH_2.N remains small and relatively constant, while the "peptid" N of the P+ and P— media regularly decreases, indicates that amino N is a very readily assimilable form, and that the amino acids and possibly the reconstructed peptids, liberated during autolysis of the fungous proteins, are quickly assimilated by the living protoplasm because they are in directly utilizable forms. The results of Zaleski and Israilsky

('14) are very significant; they found that the best source of N for yeast is the autolysate of the yeast itself. Similarly Zaleski and Pjukow ('14) showed that fungous autolysate was superior to $(NH_4)_2SO_4$ as an N source for *Aspergillus niger*.

It seems well to reconsider here the opinions of Ehrlich, Czapek, Zaleski, and others on the form in which N is directly assimilated from various N sources. While there is no conclusive proof that N obtained by the fungi from amino acids, peptids, amines, alkaloids, etc. is actually in the form of ammonia, the nicety of

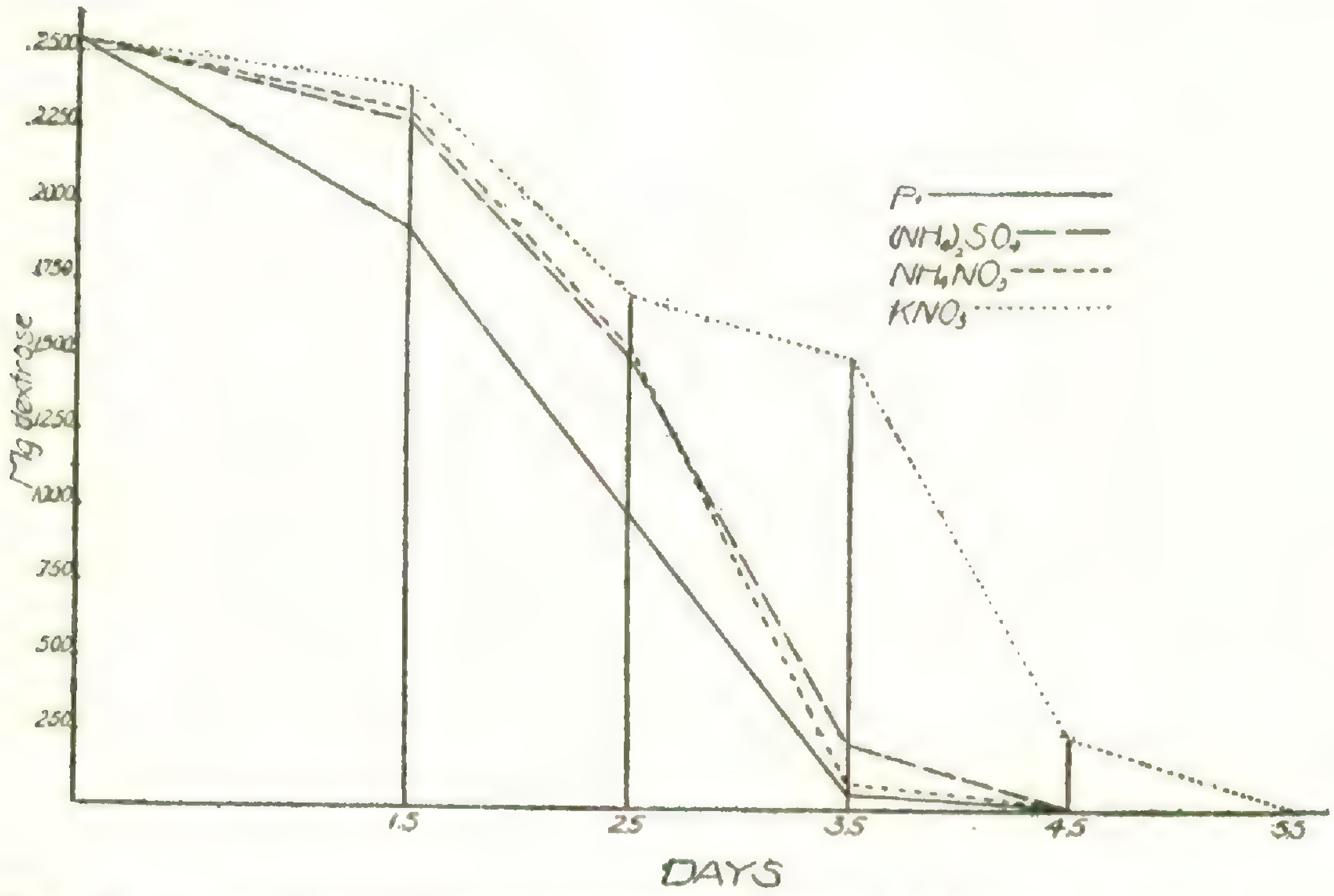


Fig. 10. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media *Aspergillus niger*, third series.

the equations developed by Ehrlich and his associates, together with quantitative proof in some cases, makes it seem likely that the NH_3 and probably its simple substitution products are the forms directly assimilated. More evidence for this is the fact that Fischer obtained the diamino acids of β vinyl acrylic acid, sorbic and fumaric acids by the direct action of ammonia on these acids. It does not require a great stretch of the imagination to think of the highly reactive cleavage products of sugars behaving similarly toward NH_3. This assuredly does not preclude Czapek's theory of the direct utilization of amino acids and other

amido compounds, if such are actual units in the protein structure of the organism involved. In fact the conceptions of both factions should be combined to help account for the various degrees of utility of a particular nitrogenous compound for different fungi. Every genus of fungi and possibly every species of a genus is an entity in itself as regards its physiology. For example, *Aspergillus niger* makes a greater growth on $(NH_4)_2SO_4$

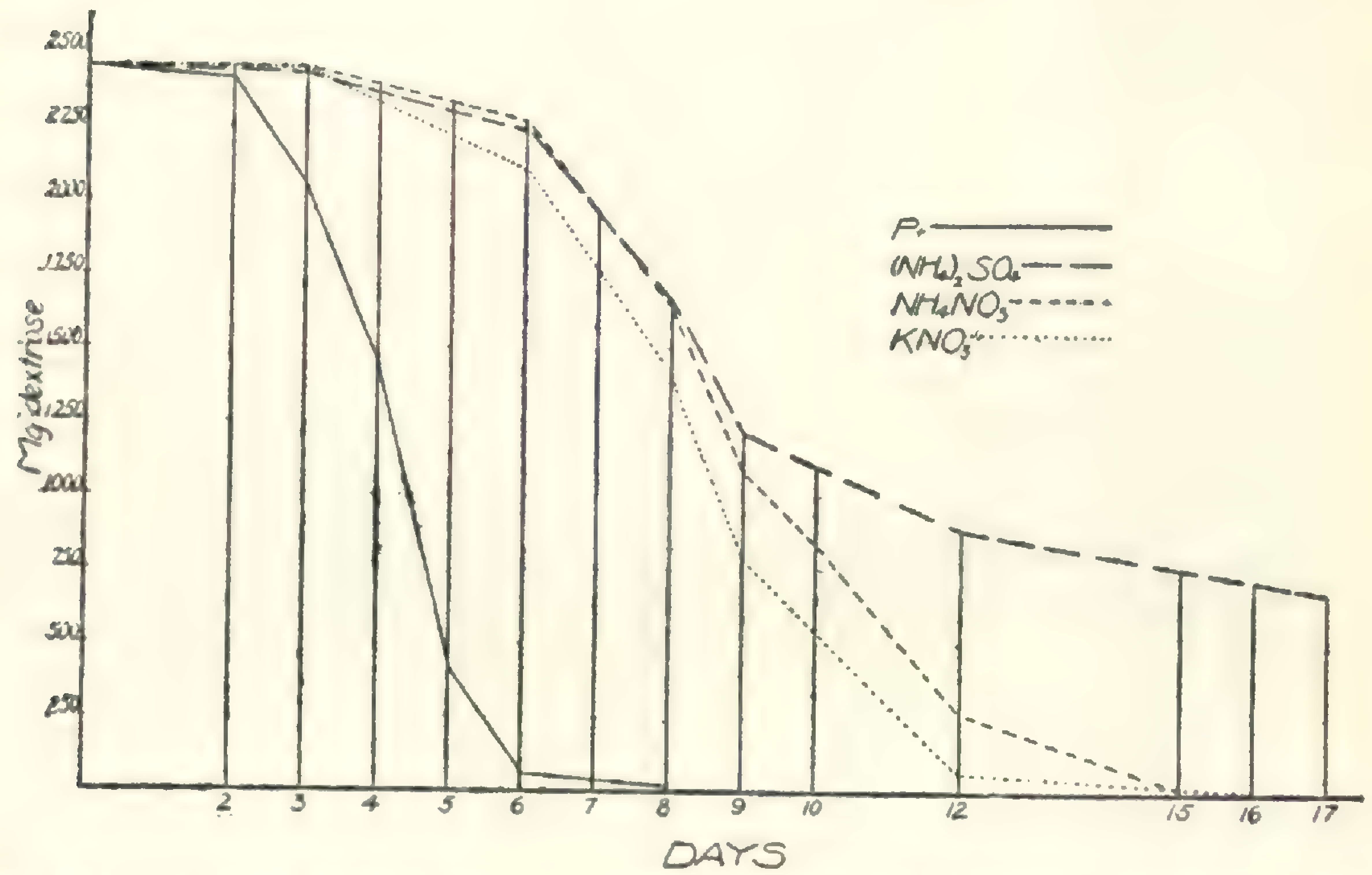


Fig. 11. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media. *Sphaeropsis malorum.*

than does *A. glaucus,* while the latter is superior in the ability to assimilate nitrates. Nitrogen distribution studies on fungous mats to discover what amino acids are actually present in the substance of the fungus, and subsequent cultural studies in which different combinations and proportions of the units are used as the sources of N would probably contribute much toward a proper understanding of metabolism (van Slyke, '11). Such work requires much time and the cooperative labor of several associates, and could not for this reason be carried out during the time of this investigation in this laboratory.

A few determinations of the total N of dry fungous mats were made and these showed the following:

Organism	Medium	Days incubation	Per cent N
Aspergillus niger	P+	4	4.85
Sphaeropsis malorum	P+	35	3.45
	P—	35	4.16
	KNO_3	35	3.813
Diplodia natalensis	P+	8	5.65
	P+	12	5.08
	P+	18	4.48
	P—	8	5.28
	P—	12	5.32
	P—	18	5.08
	$(NH_4)_2SO_4$	8	7.11
	$(NH_4)_2SO_4$	12	6.50
	$(NH_4)_2SO_4$	18	6.70
	NH_4NO_3	8	5.54
	NH_4NO_3	12	5.57
	NH_4NO_3	18	6.64
	KNO_3	8	4.60
	KNO_3	12	4.59
	KNO_3	18	4.00

The N content of the fungi is seen to vary with the medium and the duration of incubation. The percentage N in *Diplodia*

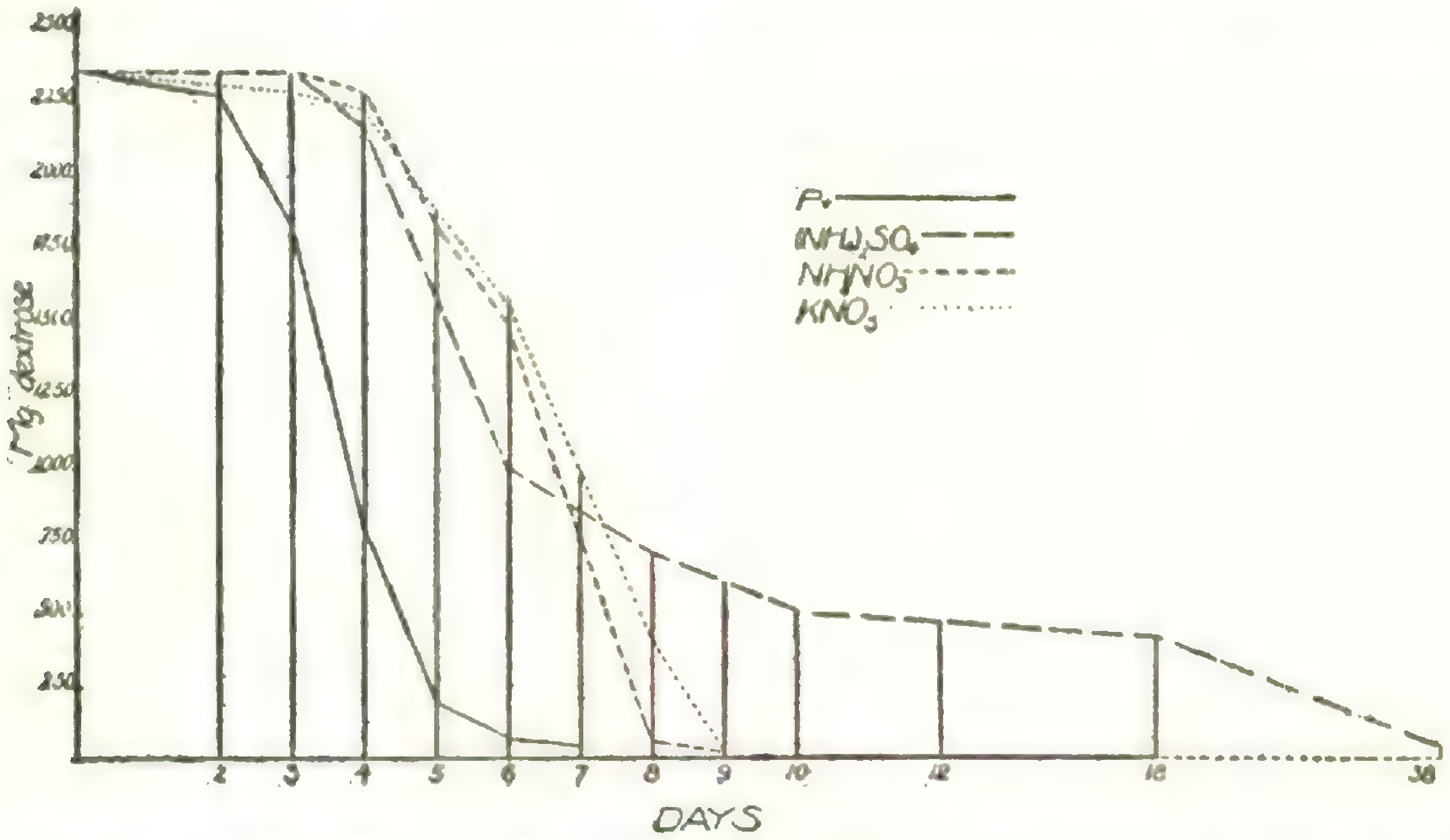


Fig. 12. Rates of carbohydrate consumption; amount of dextrose in 50 ml. media. *Diplodia natalensis.*

decreased with continued incubation on all the media except the NH_4NO_3 on which it showed an increase. This is similar to the findings of Terroine and his associates ('22) who worked with

Aspergillus niger. The media which were made strongly acid by the fungi produced mats having the highest N percentage. This is possibly due to the inhibiting action of the H ion on autolysis. The media on which the fungi made the fastest and largest growth yielded mats having the lowest per cent of N.

The behavior of the 3 fungi in the $(NH_4)_2SO_4$ medium was similar in that all increased the acidity of this solution. The greatest hydrion concentration was produced by *Aspergillus niger;* the maximum acidity (P_H 1.9) appeared in 3 days, after

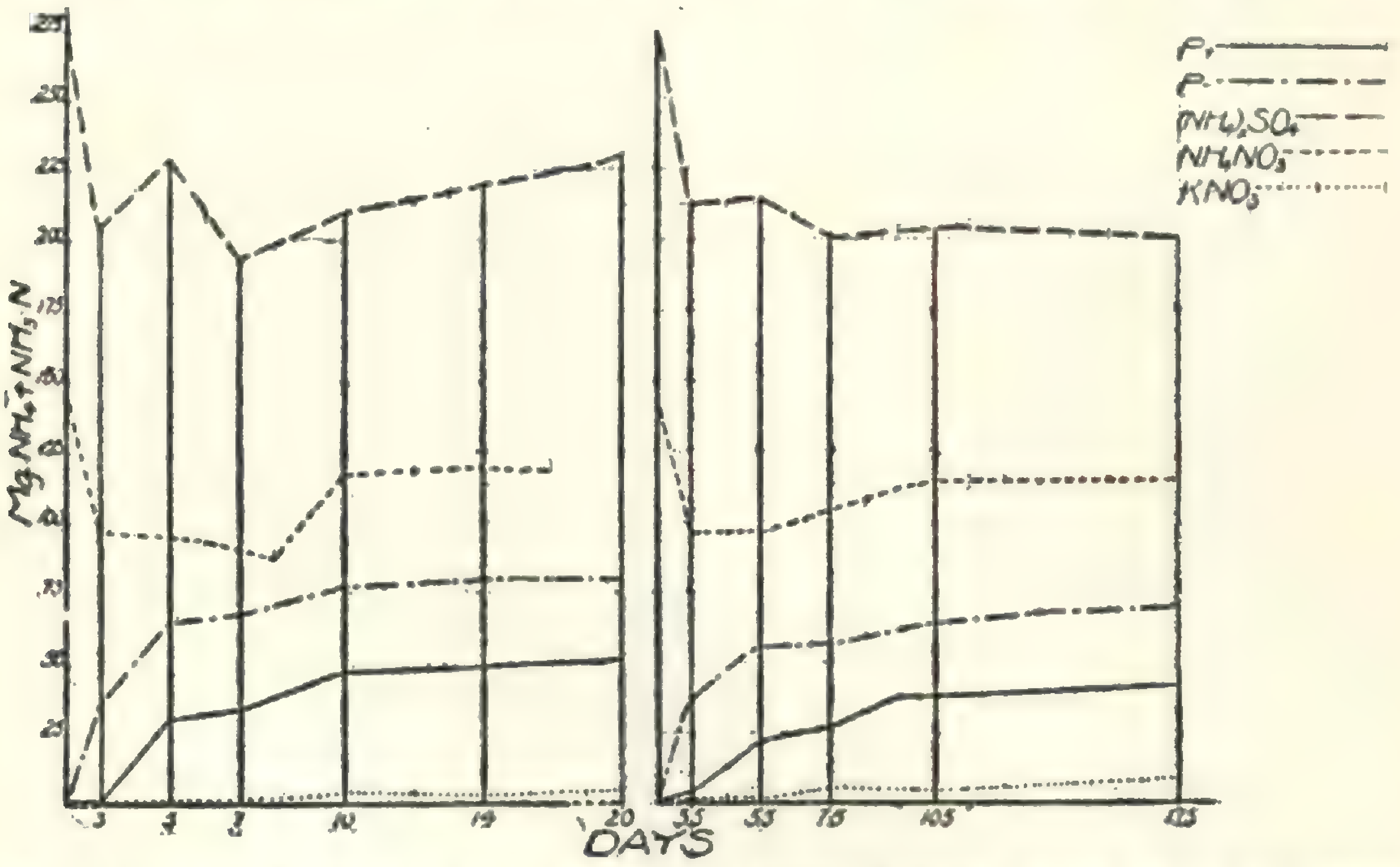


Fig. 13. Ammonium N in 50 ml. media. *Aspergillus niger,* second and third series.

which time there was a slight decrease in $[\overset{+}{H}]$ to a constant point, P_H 2.3–2.4. This decrease is synchronous with the disappearance of the sugar, the beginning of a decrease in the weight of the fungous mat, increase in the total and ammonium N from a minimum, and appearance of a trace of amino N in the medium. The maximum acidity reached is due to the sulphuric acid freed from the $(NH_4)_2H_2SO_4$ plus the organic acids formed in the decomposition of the sugar, the decrease of acidity to the consumption of the organic acids and also to autolytic processes. The iodine test for soluble starch gave a negative result after 4 days of incubation but a strongly positive test after 20 days. A similar course of reactions takes place in the cultures of *A. niger* on the

NH_4NO_3 medium. The acidity reached is P_H 1.6, greater even than in the $(NH_4)_2SO_4$ medium, indicating that the ammonia of the $NH_3.HNO_3$ is consumed more rapidly than the nitrate ion. The results for ammonium and nitrate N also show this. Boas ('18), in his criticism of Czapek's work, emphasized also the effect of reaction on the comparative assimilability of various nitrogenous compounds, but in his corrective experiments he ridiculously resorted to litmus roughly to indicate the reaction instead of determining active acidity.

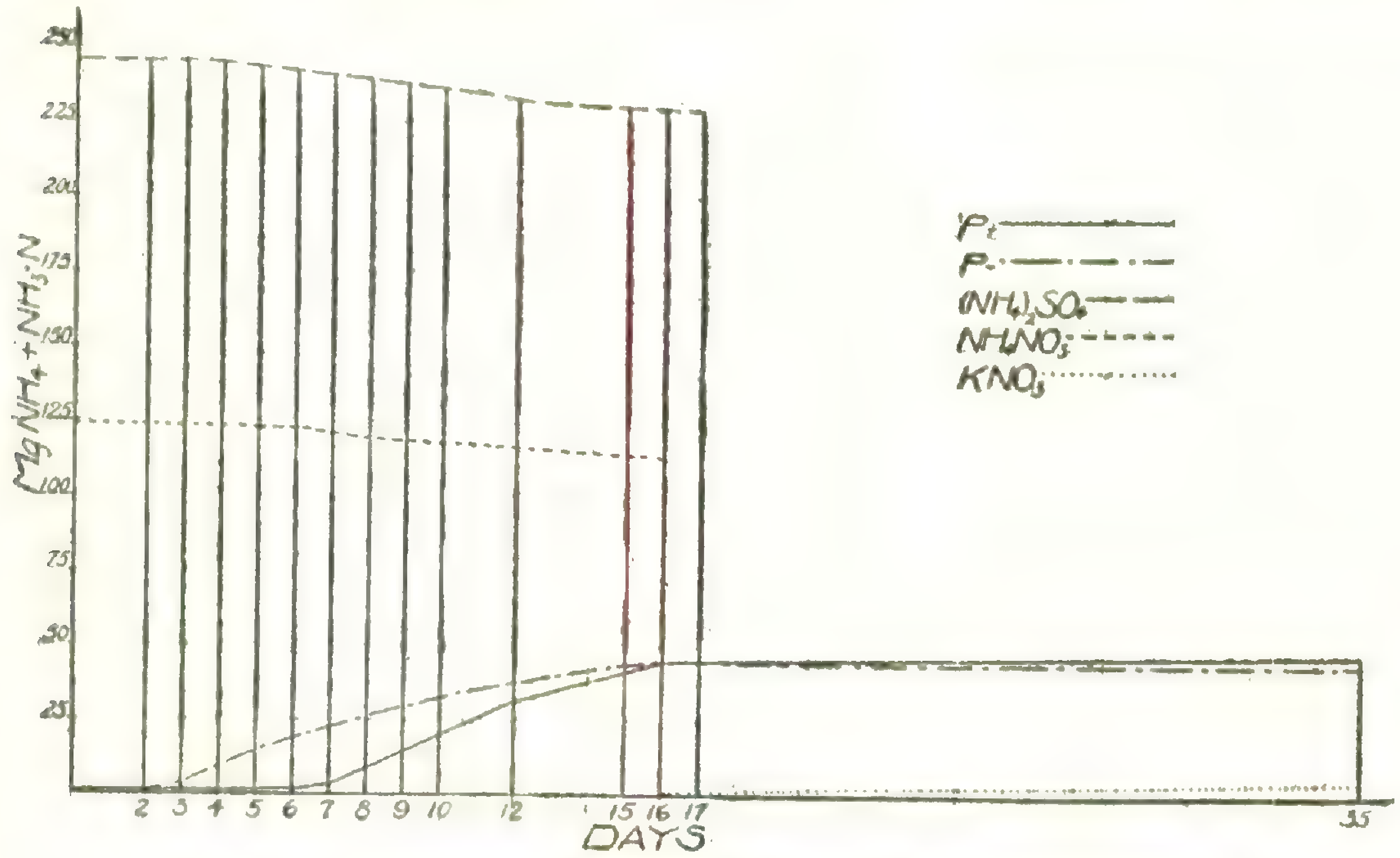


Fig. 14. Ammonium N in 50 ml. media. *Sphaeropsis malorum.*

The *Diplodia* and *Sphaeropsis* make a relatively slow growth on the $(NH_4)_2.SO_4$ medium and very gradually decrease the dextrose content of the solution, a small quantity of sugar being present even after 38 days of incubation. Similarly, the NH_4 and total N content and hydrion exponent gradually diminish throughout the entire period and the trace of $NH_2.N$ increases slightly. An appreciable odor of ethyl alcohol is evident after 7 days' growth. Autolysis in these cultures does not play an important role during the time of the experiment. These results corroborate those of Iwanoff ('21), who found that an acid reaction and the presence of alcohol arrested protein decomposition in fermenting fluids. That the courses of metabolism of these 2 fungi on the NH_4NO_3 medium are different is indicated by the

different nature of the change in hydrion concentration, the slower consumption of sugar by the *Sphaeropsis*, and the different rates of assimilation of the ammonium and nitrate ions. The *Diplodia* gradually increases the $[\overset{+}{H}]$ from P_H 4.0 to P_H 2.7 during the 38 days of incubation, while the *Sphaeropsis* during the first 6 days slightly increases the $[\overset{+}{H}]$ from P_H 4.0 to 3.8 and then during the succeeding 8 or 9 days decreases it from P_H 3.8 to P_H 5.8. The disappearance of glucose from the medium of the *Diplodia* cultures takes place about the ninth day, but not until

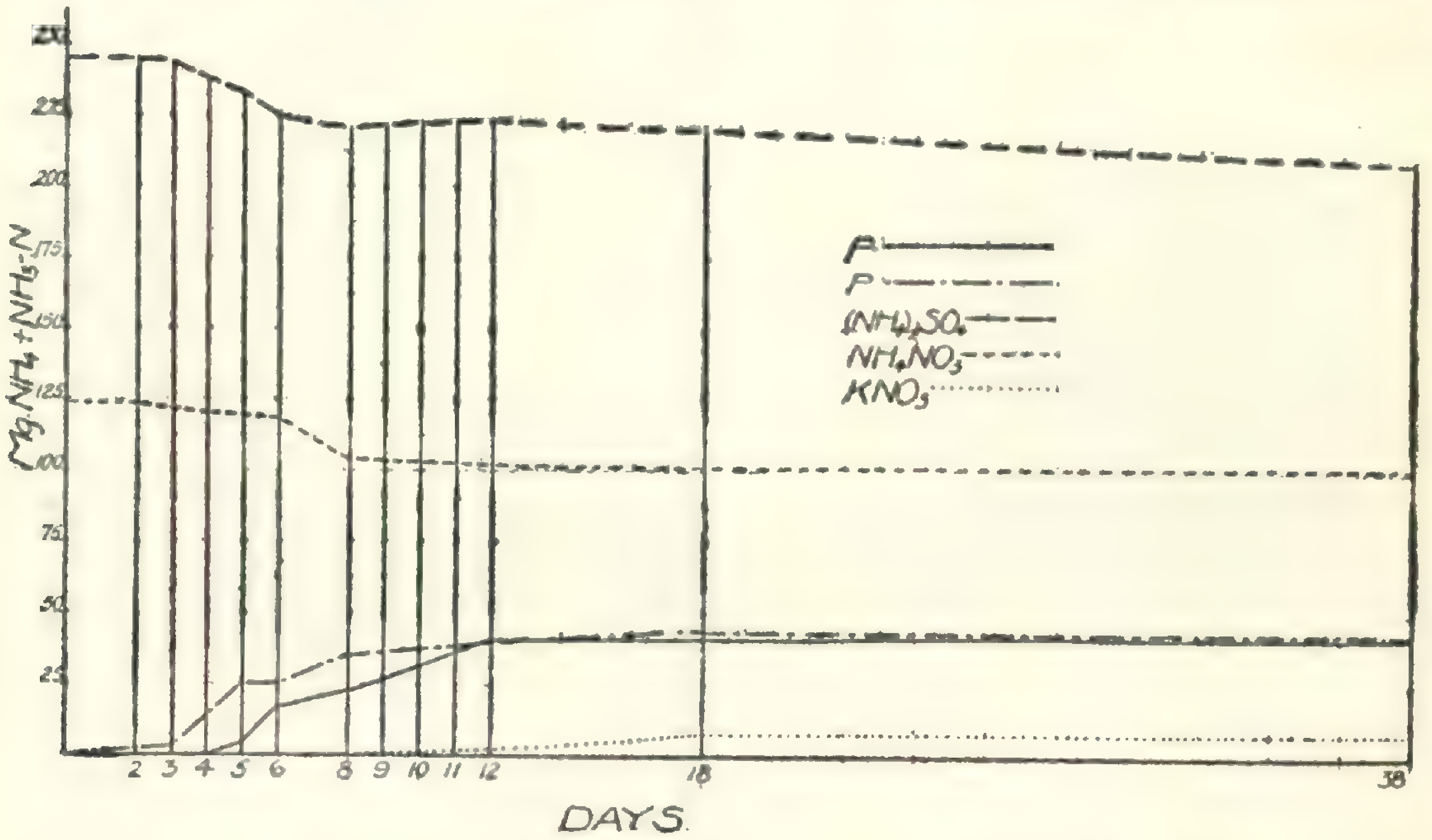


Fig. 15. Ammonium N in 50 ml. media. *Diplodia natalensis.*

the fifteenth day in the cultures of *Sphaeropsis;* and the appearance of autolytic predominance, as indicated by loss of weight, occupies the same relative position. *Diplodia* assimilates the NH_4 faster than it does the NO_3 of the NH_4NO_3 molecule, while the opposite is true for *Sphaeropsis.* This largely accounts for the different curves for active acidity. The different rate of consumption of dextrose and autolytic effects may also play some part in this change of $[\overset{+}{H}]$.

The curves for hydrion concentration of the culture solution of the second and third *Aspergillus* series on the KNO_3 medium are peculiar in that they show an increase of 0.6 P_H the first day, followed by a fall of 0.3 to 0.5 P_H during the next 4 days and then a gradual rise in P_H value to the end of the experiment. The

fall noted was not brought out by the first series with this fungus because determinations were not made until the third day of incubation. A possible explanation is similar to that offered for the action of the organism on the P— medium. The period of spore germination and extension of the young germ tubes is one in which the nitrogen of the KNO_3 is more rapidly used than is the dextrose decomposed to form organic acids, and consequently, due to the freed K, there is a slight increase of the hydroxyl ions above the original hydroxyl-ion content of the medium.

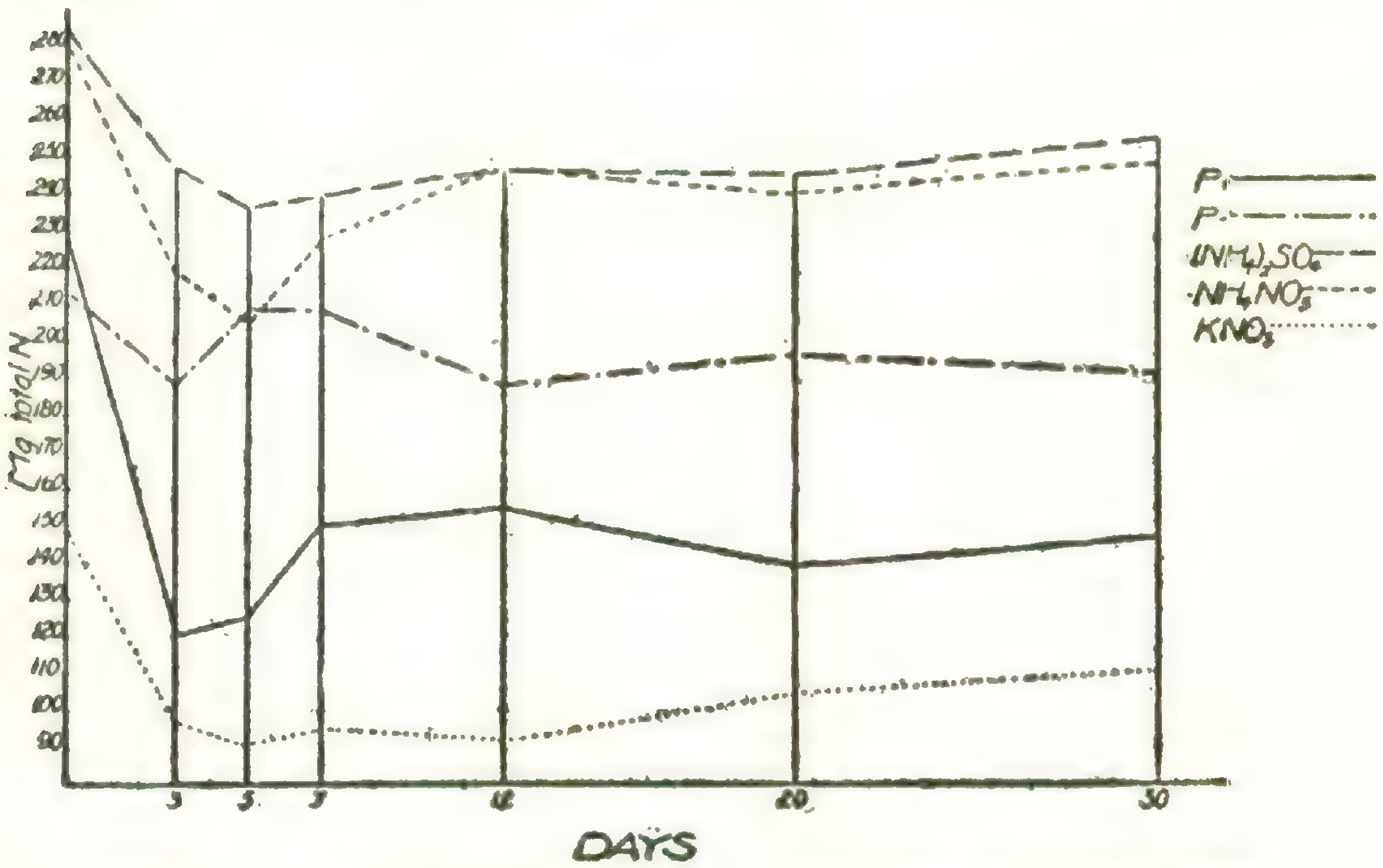


Fig. 16. Total N in 50 ml. media. *Aspergillus niger*, first series.

This is followed by the short period of the predominance of organic acids from the decomposed dextrose and the resulting increase in acidity. Then after the fifth day, the sugar and organic acids being nearly all assimilated, the increasing products of autolysis plus the OH resulting from the different rates of assimilation of the potassium and nitrate ions from the KNO_3, the K being in excess of the needs of the fungus, rapidly change the reaction toward the alkaline side. In the cultures of the slower-growing *Diplodia* and *Sphaeropsis* the above phenomenon is not in evidence. The medium was gradually diminished in acidity from P_H 4.0, becoming strongly alkaline (P_H 7.8 in the *Diplodia* cultures and P_H 8.1 in the *Sphaeropsis* cultures). Up to the twelfth day for

Sphaeropsis and the ninth day for *Diplodia* this is largely a matter of the differential absorption of the K and NO_3 ions. When the sugar has been consumed the dry weight of these organisms immediately begins to decrease, and autolytic products rapidly change the reaction to strongly alkaline (see page 317 of this article for Waksman's explanation). The total N (as applied to cultures of *Actinomyces*) of the culture medium decreases slowly but simultaneously with the rapid diminution of glucose, and this rapidly rises above the minimum when the sugar has dis-

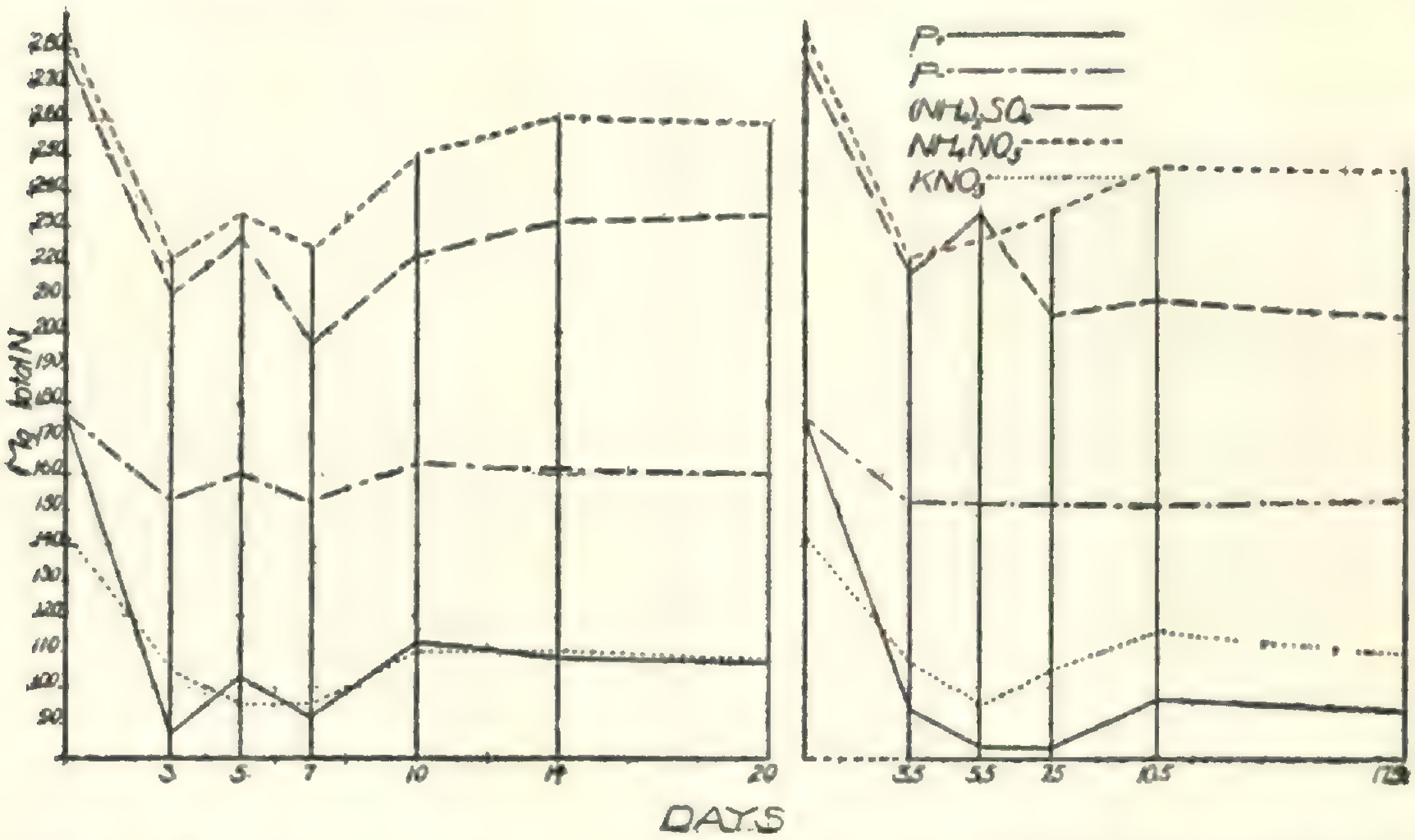


Fig. 17. Total N in 50 ml. media. *Aspergillus niger*, second and third series.

appeared. On continued incubation there may be a fall in the total and NH_4.N content due to the precipitation as $MgNH_4PO_4.6H_2O$ of NH_3 formed in autolysis; this is observable in the results for the *Diplodia* series.

The determinations show that in all the cultures of all 3 fungi on the inorganic nitrogenous media the amino N content of the solution did not attain more than a trace; 7.26 mgm. per 50 ml. of medium was the greatest value determined. This in itself indicates the ready assimilability of this form of nitrogen. Consider, for example, the 35-day cultures of *Sphaeropsis malorum* on the KNO_3 medium. Autolysis had decreased the weight of the fungus to almost one-half of the maximum weight attained in

10 days of incubation, yet the NH_2.N content of the culture fluid was only 0.14 mgm. per 50 ml.

To recapitulate, it is seen that the data throw some light on the forms of N which are directly utilizable and most serviceable for the fungi. Judging by the maximum dry weight attained, and disregarding the effects of acidity and other factors on metabolism, peptone in the presence of dextrose was the superior N source for all 3 fungi, whereas peptone in the absence of suga

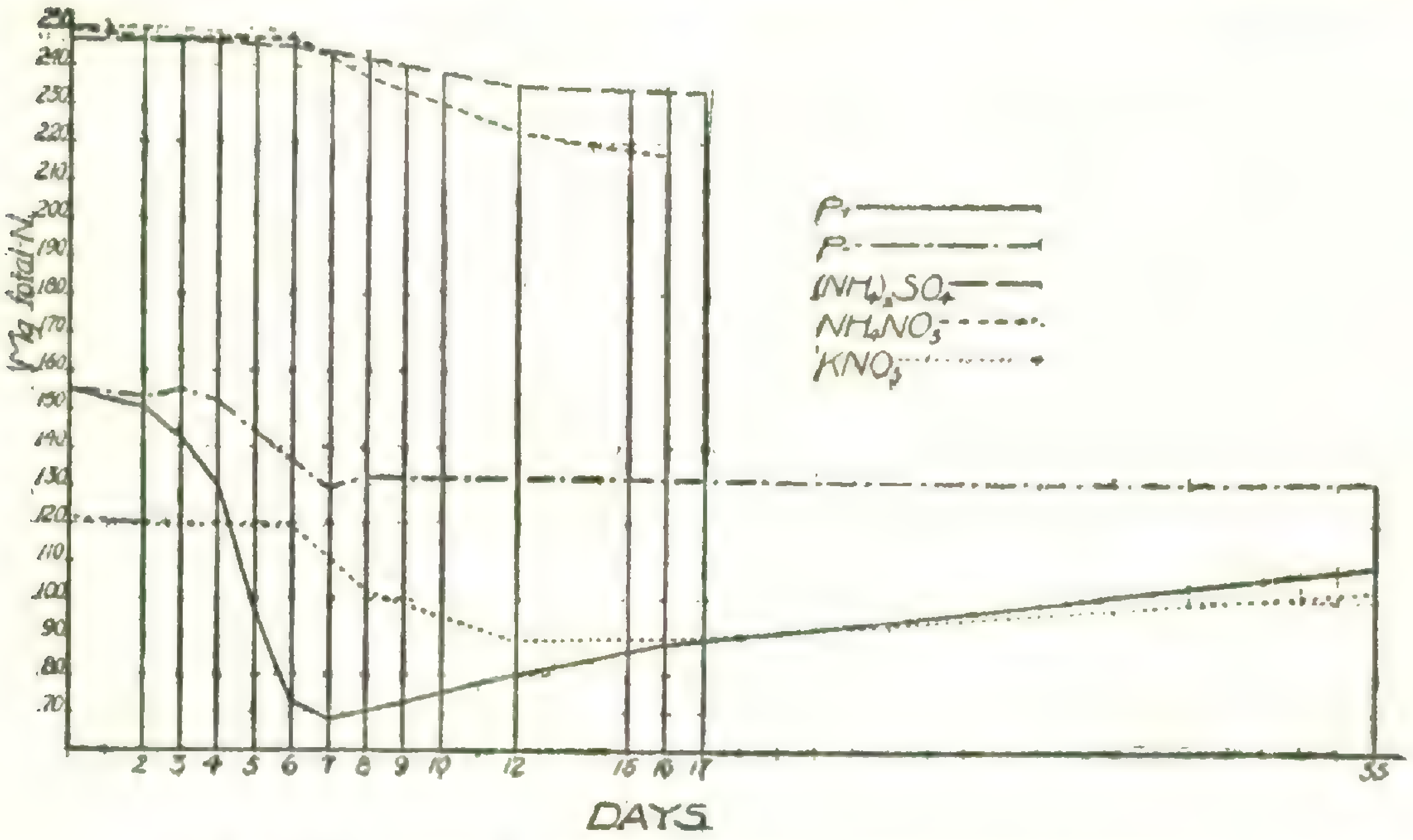


Fig. 18. Total N in 50 ml. media. *Sphaeropsis malorum.*

was distinctly inferior. This supports the point brought out on pp. 350–351 that amino acids and even "peptid" units may be directly assimilable if they are actually found as such in the proteins of the fungi. To this must be added that an available carbon source must be present to supply readily the energy necessary for the cementing of these building stones. It is evidently difficult to obtain this energy by deamidization of the peptone components and utilization of the non-nitrogenous complex. For the same reason amino acids serve but poorly as sources of both C and N, as has been shown by several investigators. However, it is probable that the NH_2 group of the amino acids is a very readily available form for those organisms that possess deamidizing enzymes sufficiently strong to split off this group readily. Waksman ('18) found indications of such en-

zymes in *Aspergillus niger* and other fungi. The theoretical equation for this would show the NH_2 group hydrolytically split off as ammonia, leaving the hydroxy group in the corresponding place in the acid molecule, as was pointed out by Ehrlich. Ammonia thus formed is not detectable; therefore, it seems reasonable to assume that it is not formed, and that the NH_2 group is united directly to the non-nitrogenous units, the excess hydrogen increasing the acidity of the solution. The initial rise in H-ion concentration of all the P+ cultures may be partly due to this as well as to organic acids from the sugar.

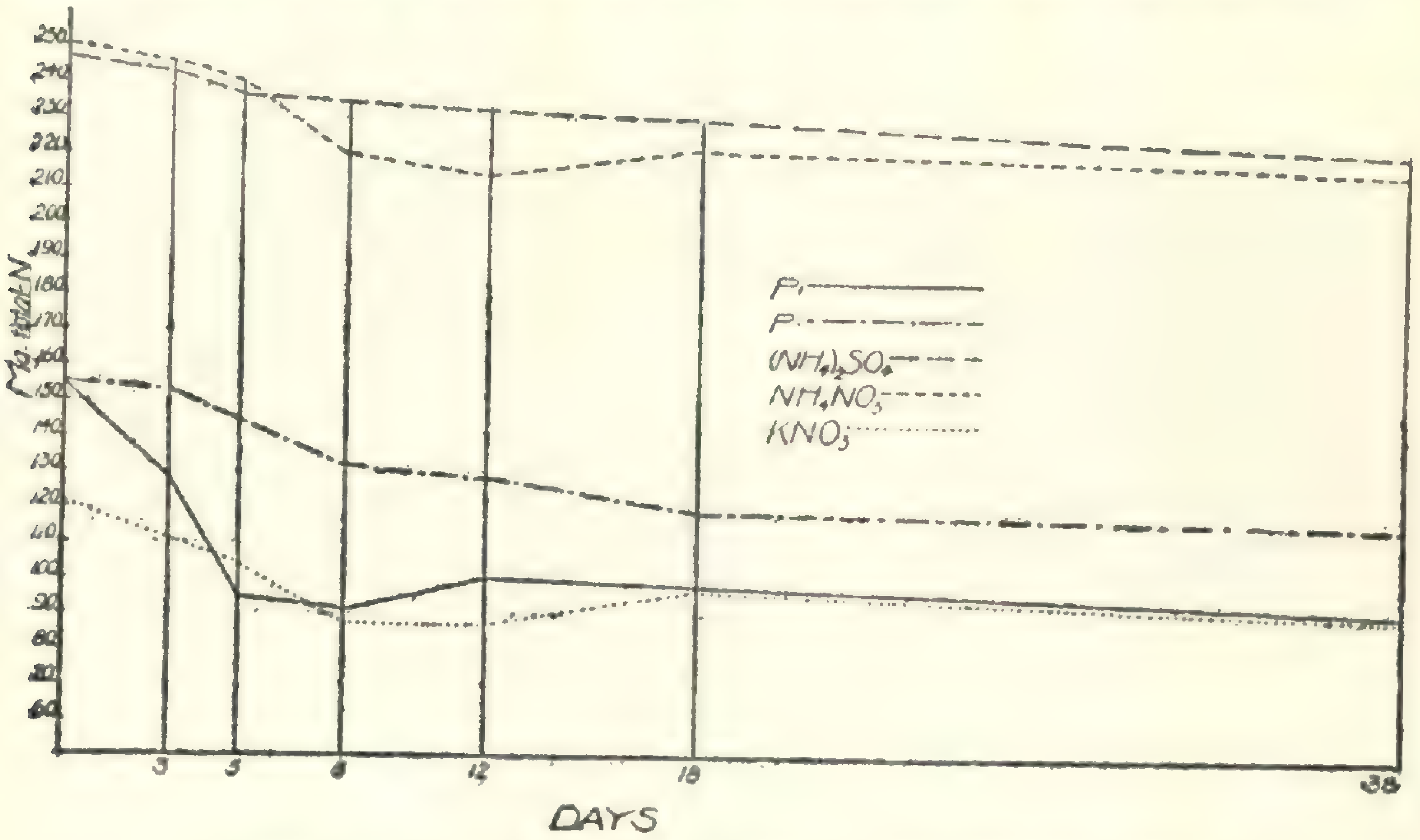


Fig. 19. Total N in 50 ml. media. *Diplodia natalensis.*

For *Aspergillus niger* the NH_4 ion is more serviceable than the NO_3, as the results show greater absorption of the former than the latter from NH_4NO_3. And judged by the weight of the fungus the $(NH_4)_2SO_4$ is superior to KNO_3 in spite of the acidity produced as a result of the use of the first named. *Sphaeropsis malorum* in the NH_4NO_3 medium absorbs the NO_3 ion at a slightly greater rate than the NH_4, and the organism on the KNO_3 medium makes a faster and larger growth than on the $(NH_4)_2SO_4$, showing that it is more sensitive to the free H_2SO_4 than is the *Aspergillus.* Judged by the rate of absorption of the ions of the NH_4NO_3 medium, the *Diplodia,* on the other hand, shows a slight preference for NH_4. The sensitiveness of this organism to the hydrion,

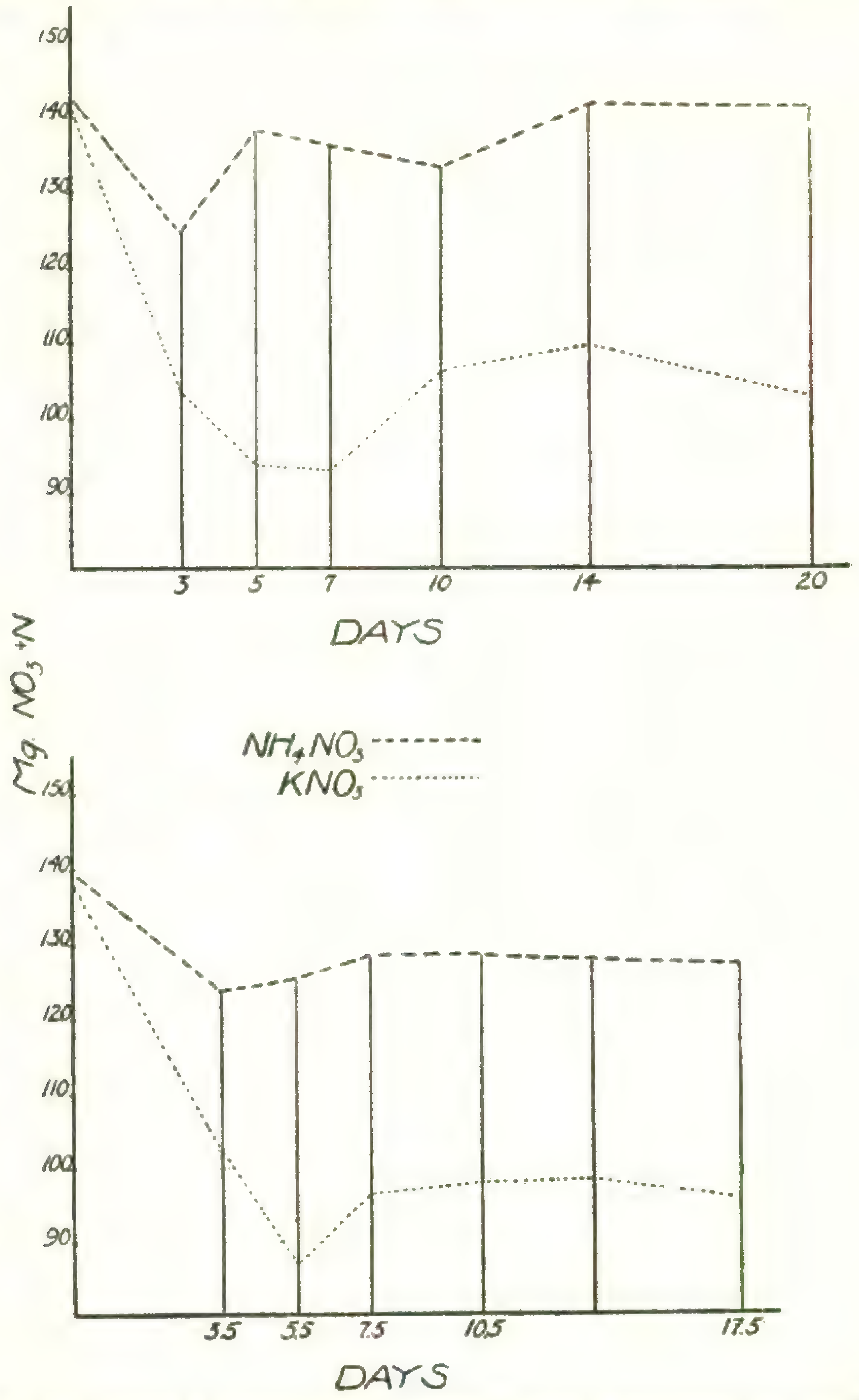


Fig. 20. Nitrate N in 50 ml. media. *Aspergillus niger*, second series (top; by difference); and third series (bottom; Strowd's method used).

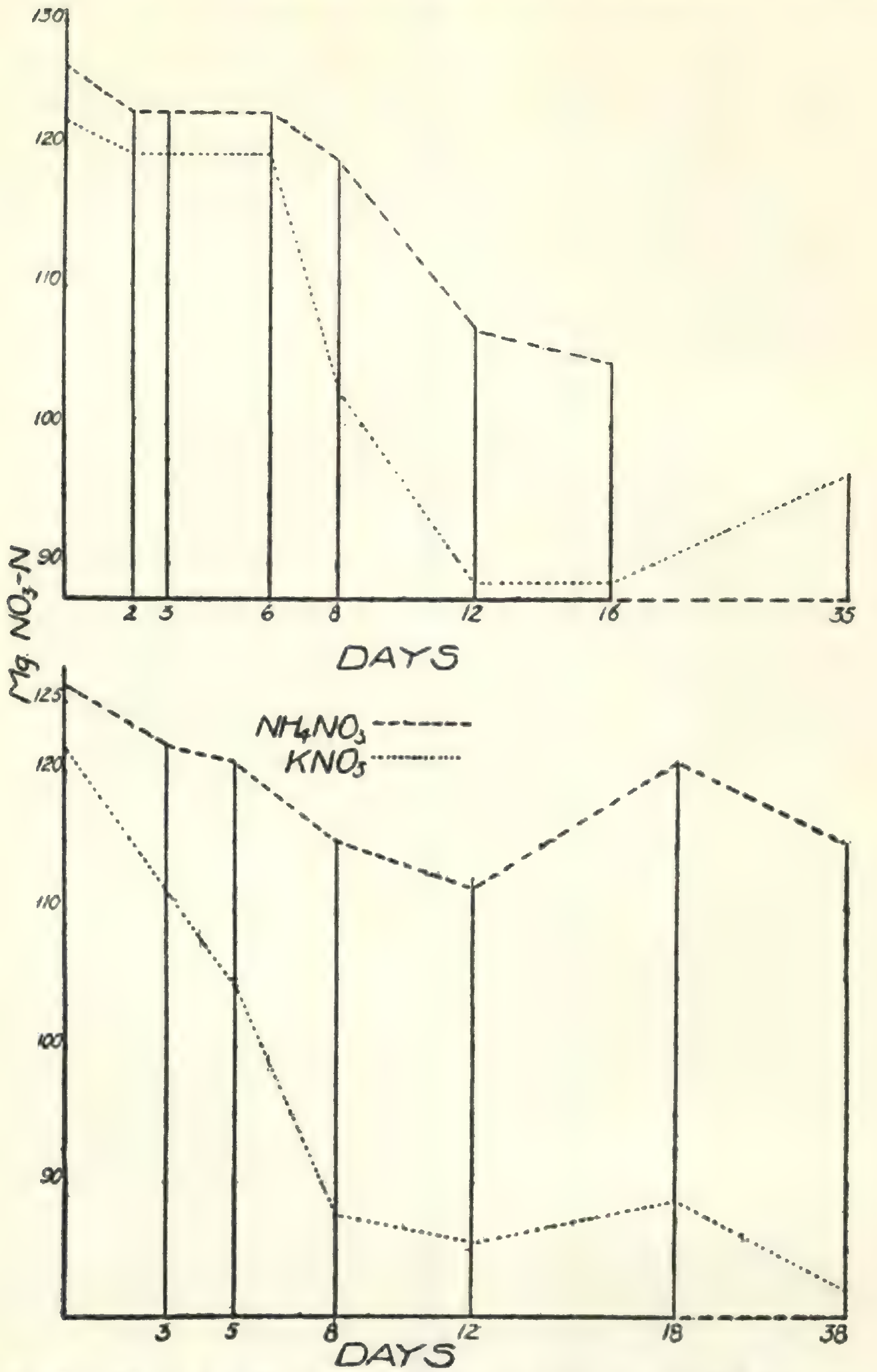


Fig. 21. Nitrate N in 50 ml. media. *Sphaeropsis malorum* (top; NO_3.N by difference); *Diplodia natalensis* (bottom; NO_3.N by difference).

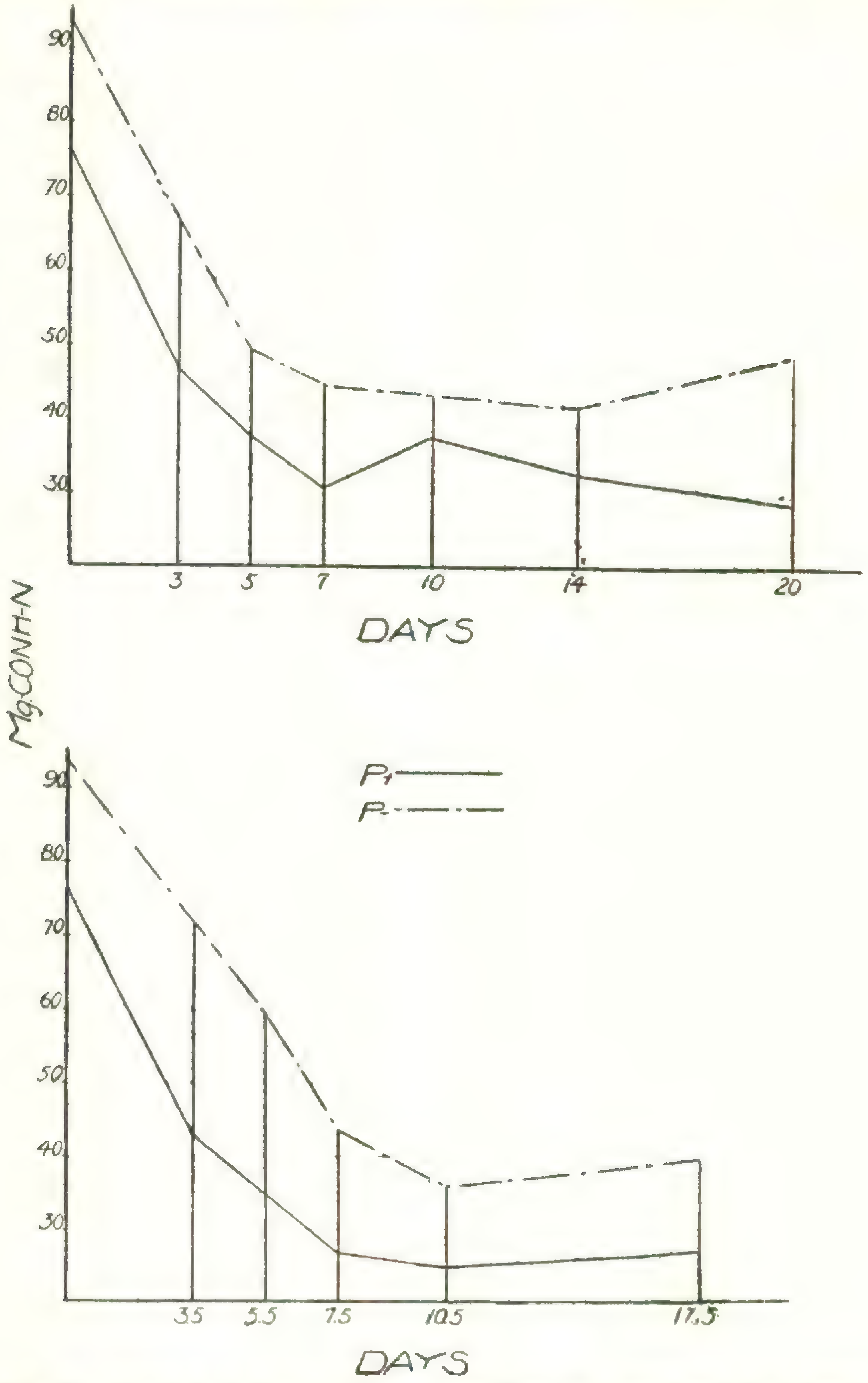


Fig. 22. Peptid N in 50 ml. media. *Aspergillus niger*, second and third series.

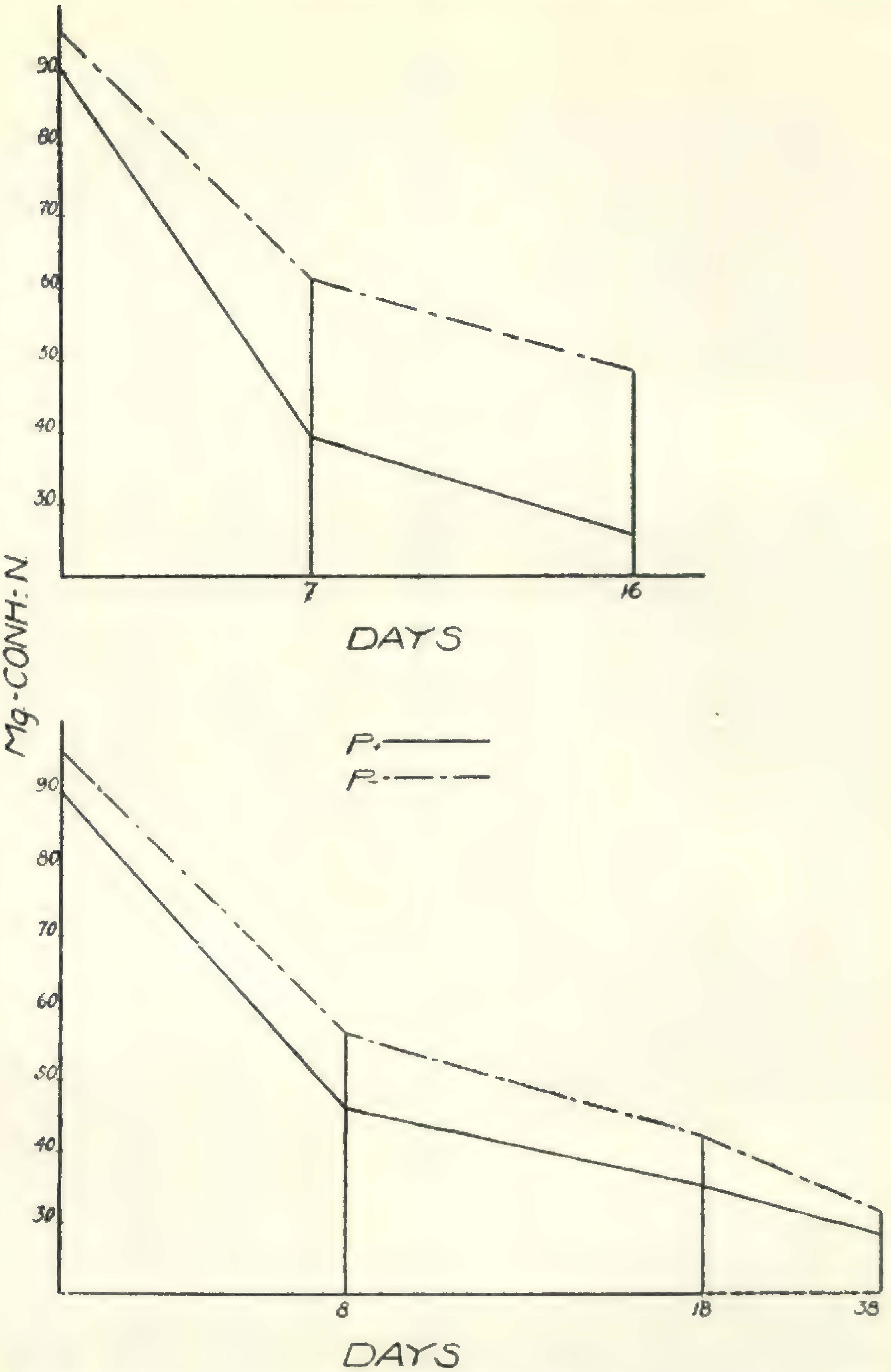


Fig. 23. Peptid N in 50 ml. media. *Sphaeropsis malorum* (top); *Diplodia natalensis* (bottom).

as shown by its growth on $(NH_4)_2SO_4$, is similar to that of the *Sphaeropsis*.

The general conclusion from these considerations is that there is a distinctive physiology for each of these 3 organisms and that this conditions the form of the inorganic nitrogen which is most assimilable. There are no conclusive data in the literature to show that nitrates must be reduced to nitrites or ammonia before assimilation. Reasoning *a priori*, the form of nitrogen supplied must be reduced (if an oxide) or oxidized (if NH_4 or NH_3) to form the amino group, and other conditions (as H-ion concentration) being the same, organisms show a differential use of these ions because of their different powers of reduction, oxidation, and synthesis. As has been already pointed out, many erroneous assumptions have been made in this regard because investigators failed to consider the NH_3 produced proteolytically. For this reason also, the necessity for making dry-weight determinations at frequent intervals is evident.

The order of relative assimilability of nitrogenous compounds for a specific fungus at one H-ion concentration may be entirely different from the order at another P_H value. Similarly the effects of temperature, humidity, light, aeration, agitation, and possibly other factors on assimilation must be so considered; the problems are complex.

Summary

1. A review of the literature on the N metabolism of fungi is given.

2. The methods used are described and reasons are given for the selections made.

3. Autolysis is at first indicated by decrease in dry weight of the fungous mat from a maximum and by the formation of ammonia in the peptone and KNO_3 media; somewhat later, by increase in total N of the culture solution from a minimum in all the media, and by the appearance of a trace of amino N in the 3 inorganic nitrogenous media.

4. Autolysis in a species is proportional to the rate and amount of growth attained.

5. Ammonia is the chief nitrogenous product of autolysis and is a waste product of the splitting of the peptone of the media in the absence of another C source. In the presence of dextrose NH_3 was reassimilated. Disappearance of carbohydrate from the culture medium is synchronous with the beginning of autolysis.

6. In cultures whose H-ion exponent becomes greater than P_H 7.0 the loss of N is due to the evolution of NH_3.

7. Conditions necessary to the formation of crystals of $MgNH_4PO_4.6H_2O$ and $Mg_3(PO_4)_2.4H_2O$ in the cultures were determined.

8. The probable causes for the shifting of the H-ion concentration of the media are discussed.

9. Nitrogen of the amino group is readily assimilated by the fungi studied.

10. Some factors influencing the N content of the fungous mat are the N and C sources of the medium, the length of incubation, rate of growth, and hydrion concentration.

11. The organisms displayed markedly different physiological relations; this was indicated by their rates of growth and of sugar consumption, by their utilization and excretion of the several forms of nitrogen, and by the varying nature and extent of H-ion change of the medium.

The writer here expresses his appreciation to Doctor B. M. Duggar for criticisms and suggestions given throughout the work, and to Doctor George T. Moore for the privileges and facilities of the Missouri Botanical Garden.

Graduate Laboratory, Missouri Botanical Garden.

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AN HISTOLOGICAL STUDY OF REGENERATIVE PHENOMENA IN PLANTS[1]

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Introduction

Nemec ('05), Pfeffer ('05), and Prantl ('74) employ the word regeneration in its narrowest sense when they consider that it is the formation of new parts exactly alike in number and position to the organs injured or removed. Since new structures generally originate only from actively growing tissues, regeneration is practically limited to the embryonic tissues of the root and shoot. For example, if an old root tip is removed, the new root tip is regenerated. Roots forming on the stem, however, are not regenerated roots but simply adventitious roots. An example of this type is the root developed at the nodes of *Tradescantia* when that plant is placed in a glass of water. The extreme opposite of this view has been held by Vöchting ('78), Goebel ('02), Morgan ('01), and Klebs ('03) who consider the development of dormant buds present on the part before injury to be a regeneration phenomenon. They, therefore, include in their definition of regeneration a phase of normal vegetative growth which might be termed merely the stimulation of bud development, the production of new roots or new buds, etc., in any position in which these organs do not normally occur. A more moderate view, however, is assumed by Miss Kupfer ('07) who says that regeneration "ought to be limited to organs formed 'de novo' at a place or under conditions not normally so [formed]." Therefore, she excludes latent root and shoot development which occur, for example, when a willow twig is placed in the ground, and as would be included in the definition by Goebel ('02), Vöchting ('78), and Morgan ('01).

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University and submitted as a thesis in partial fulfillment of the requirements for the degree of master of arts in the Henry Shaw School of Botany of Washington University.

Summary of Literature as Regards Tissues Involved in Regeneration

The work on regeneration has dealt primarily with the organographic aspect and also with the theories as to the cause of the releasing stimuli in the phenomena. Some studies have laid stress upon the external influences to which the plant has been subjected and the relation of these to the production of regenerative processes; likewise, the tendencies inherent in the plant itself have been duly considered. Therefore, a relatively small amount of literature deals with the tissues concerned in regeneration. Prantl ('74) found that in the case of corn and *Vicia Faba* regeneration was effected through the intermediary of a common callus which represented the passage of the different systems into one another. Moreover, he found no differentiation in the callus tissue of specific groups representing the diverse tissues of the root. In 1900, and later in 1902 and 1903, Goebel conducted elaborate experiments on *Taraxacum, Bryophyllum crenatum,* and other plants. His conclusions were concerned chiefly with the physiological characters of regeneration, its causes and effects. Simon, in 1904 and later in 1908, after investigating the regeneration of root tips and shoots, stated that all tissues may indirectly form a callus and then regeneration may take place or, directly, each tissue may form a callus. McCallum's work in 1905 on species of *Phaseolus, Salix, Helianthus, Taraxacum,* and other plants was of a physiological nature, dealing with the disturbances in nutrition, disturbance in water content, wound stimuli, and correlation. Nemec ('05) made an elaborate study of the regeneration of roots and found regenerated parts developed from either a callus formation or directly from the division of the cells at the base of the dermatogen. Miss Kupfer ('07) found that regeneration of fleshy roots developed from the cambium and callus, as in the horseradish, sweet-potato, and parsnip. She makes no reference to the sectioning or preparation of slides of her material. Therefore, from these studies, organ regeneration is dependent upon the cambial and callus tissues.

Experimental Work

Object.—The object of this work is to secure regeneration, to find the earliest stages of the divisions of those cells giving rise to the regenerated parts, and to trace the development of those tissues and the relation of those regenerated parts to the tissues of the original plant by means of histological study.

Methods and materials.—The materials used were flax seedlings, pieces of sweet-potato, horseradish, parsnips, tobacco stems and buds, and *Bryophyllum* leaves. The work was carried on with sterile sand cultures, water cultures, and potted cultures. Care was taken to use sterile cultures that the materials might be kept a longer period of time than in previous studies free from molds, bacteria, etc. Sterile cultures necessitated the use of nutrient solutions. The nutrient solutions used were Shive's solution (A) and Duggar's solution (B).

The following cultures were set up in test-tubes, Ehrlenmeyer flasks, and quart jars, all being plugged with cotton and then sterilized in the autoclave at 15 pounds pressure for 20 minutes. Tumblers were inserted over the jars that no dust might sift through the cotton. The test-tubes were kept in a moist chamber to keep them free from dust.

FIRST SET OF EXPERIMENTS

Experiment 1.—Ordinary tap-water was used for the water cultures. A 3-inch piece of a horseradish root was cut at the top and bottom and suspended in a tumbler of water by means of a wire. In about a week roots and shoots developed. Upon examination they were found to have developed from the cambium.

Experiment 2.—A piece of a horseradish root was peeled so as to leave nothing but the pith. This was suspended in a tumbler of water which was continually refilled. After 9 days a few regenerated shoots developed, but upon examination they were found to have originated from a few adhering cambium cells.

Experiment 3.—A whole sweet-potato was suspended by a wire in a quart-jar of water. Within 10 days many shoots developed normally from the cambium.

Experiment 4.—The pith of a sweet-potato, similarly suspended in a jar of water, exhibited no regeneration even after 2 months. Wherever regeneration occurred in any of these cultures, the regenerated parts were cut out with as little of the root proper as was necessary and put in killing solutions as later described in preparation for histological study. Even those portions which gave only a very slight indication of the possibility of regeneration were used.

SECOND SET OF EXPERIMENTS

About 10 cc. of sand were added to each of 4 test-tubes. To 2 was added solution A and to the other two, solution B. These were sterilized and, according to the preparation of sterile cultures, portions, about 1 cm. square, of tobacco leaves and buds were added. No regeneration occurred even after one month. Similar cultures, using pieces of sweet-potato instead of tobacco, were set up, but these, too, exhibited no regeneration. These same experiments were repeated a few days later but with no results. Either the sections were too small or the nutrient solutions were lacking in some way or some other factor prevented the regeneration which, according to Miss Kupfer, developed under non-sterile conditions. A few weeks later similar experiments were set up, using tobacco leaves and buds and pieces of sweet-potato. In this case, however, a few cc. of 1 per cent cane sugar and 1 per cent peptone solutions were added to the nutrient solutions. No regeneration occurred, and this brought up the possibility of using larger pieces. Accordingly, pieces of parsnip, horseradish, and sweet-potato, about 1 cm. in thickness, were placed in sterile sand cultures in ordinary quart-jars. These jars were plugged with cotton and over them tumblers were inserted. To these solutions were also added a few cc. of 1 per cent peptone and 1 per cent cane-sugar solutions. The radish and potato roots were soaked in Javelle water for 2 hours before sectioning, to render the outer surfaces sterile. Within about a week all pieces developed regenerated roots or shoots. The early stages of these regenerated parts were removed in as small portions as possible and fixed for microscopic study.

THIRD SET OF EXPERIMENTS

(1) An entire horseradish root was covered with soil.

(2) A root of the horseradish was peeled down to the pith and covered with soil.

(3) Horseradish roots were cut in half longitudinally and horizontally and were placed in the soil.

(4) Similar pieces of the sweet-potato were used.

(5) The peelings of the radish and potato were planted. After one week all pieces, except the peels, exhibited regenerated roots or shoots. As previously described, these parts were removed, fixed, and preserved for study.

(6) Four detached *Bryophyllum* leaves were placed in a moist chamber on wet sand with the bases of the petioles covered. Every notch exhibited regenerated shoots and roots. The earliest stages of these were cut off and killed.

Perhaps the most interesting regeneration was developed by the flax seedlings. The flax seeds were repeatedly planted in porous saucers containing a mixture of sand and soil, and kept in moist chambers. After the unfolding of the cotyledons, these seedlings were decapitated about 1–2 cm. beneath the cotyledons. In some instances where the roots were above the soil, these too were cut off. After 6 to 10 days, each stem exhibited tiny swellings, as many as 8 appearing on one stem. Soon these swellings developed into shoots. When the root was cut off, these swellings appeared at the base of the stem and soon developed into new roots. As many as 4 regenerated roots developed on one plant. The stems that exhibited swellings and regenerated roots and shoots were cut into small sections so that each section had one swelling or regenerated root or shoot. These sections were then fixed as described below.

After washing, the materials were dehydrated, infiltrated, sectioned, and mounted in balsam according to the method given in Chamberlain's 'Methods in Plant Histology.' Some of the sections were stained in Delafield's haematoxylin and some in saffranin.

As stated before, after some regeneration was evident with the hand lenses, those sections and those giving slight indication of the possibility of regeneration as described before were killed with the following solutions:

Materials	Solution	Time	Wash
Flax	Chromo-acetic (0.5 gm. chromic acid 1.0 cc. glacial acetic 100 cc. H_2O)	12 hr.	12 hr. running H_2O
Flax Sweet-potato Horseradish Parsnip	Corrosive sublimate + acetic acid (1.2 g. sublimate 3.0 cc. glacial acetic 100 cc. alcohol of, (a) 5% or (b) 15% or (c) 95%)	3-4 hr.	12 hr., (a) 5% alcohol or (b) 15% alcohol or (c) 95% alcohol

The plates, with the exception of pl. 15, illustrate the histological study. Plate 15 shows: (1) a normal flax seedling, one with decapitated roots, and one with the cut-off cotyledons, with the regenerated shoots and roots as a result of decapitation; (2) the notches of *Bryophyllum* leaf were the places at which occurred regeneration; (3) a sweet-potato developed roots from the cambium on the cut surface and normally through the cortex and epidermis; (4) the horseradish exhibited a similar condition to the potato.

Plate 16 shows: (1) a cross-section of a flax stem with the central cylinder of phloem and xylem cells but no well-defined cambium tissue, the large irregular cortex cells, and the small, more regular epidermal cells; (2) *b* represents the earliest stages in the division of the epidermal cells to form a swelling; *c*, *d*, and *e* show the continued divisions to form a larger swelling, which finally would result in the formation of a true bud which would then seek direct connection with the central cylinder of the stem. Thus, the origin of the regenerated shoot in the flax is from the division of the epidermal cells followed by the division of the cells thus formed. There is the possibility that if there were a definite cambium that regeneration would originate from those cells, as in the radish, parsnip, etc., when the cambium is present and, undoubtedly give rise to the regenerated parts.

Plate 17 shows the regenerated root of the horseradish and the regenerated bud of the sweet-potato. Because the sections of both the potato and radish exhibited similar regenerative phenomena, only one case from each has been drawn: *a* shows a well-

differentiated group of meristem cells within the section of the radish. The origin of these cells is not shown, but it is plain that it cannot be from the epidermis or cortical layer, since these layers completely enclose the regenerating tissues; *b* shows a similar group of regenerating cells with a direct connection to the cambium of the root. Since, then, this group shows this connection, it seems that its origin must be from those cells or the cambium. *c* shows the bud enclosed in a similar manner. Other slides exhibited buds likewise enclosed by the epidermis and cortex and with a direct connection to the cambium. Hence, shoots, too, must originate from the division of cambium cells.

Sections through the vegetative points of the *Bryophyllum* leaf are shown in pl. 18: *a* represents a section through a normal portion showing a vein; *b* is an enlarged section of the vein with the division of the small phloem cells; *c* shows that division carried still farther until it disfigures the leaf by a tiny swelling, which results, as in *d*, in the formation of a regenerated root and shoot. Thus the root and shoot arise from the division of the small phloem cells of the vein near the vegetative points or notches of the leaf.

General Results

After about 5 or 10 days, there appeared roots and shoots on the horseradish, parsnip, and sweet-potato in the sterile sand cultures. Buds appeared after 10 days on the radish and potato cuttings which were first washed in Javelle water. Similar results were noted in the water cultures and sand cultures. After the cotyledons of the flax seedlings had been removed, there appeared tiny buds of shoots on the stem, and when the roots had been removed new roots were regenerated (pl. 15). Every notch of the *Bryophyllum* leaves regenerated new roots and shoots. The tobacco cuttings failed to regenerate and decayed. It was difficult to render them sterile on account of the many hairs on the surface. Freehand sections were made of the parsnip, horseradish, and sweet potato, and stained with iodine. It was plainly evident that the regenerated part was connected with the cambium, the cells of which took on the characteristic color of

protoplasm stained with iodine while the surrounding cells were filled with purple-stained starch grains (pl. 15). From the prepared sections, it can be seen that, according to pl. 17, the regenerated parts of the potato and radish came from tissues other than the epidermis or cortex. The one drawing on this plate shows the definite connection with the cambium. The parsnip slides exhibited an exactly similar condition. Regeneration in the *Bryophyllum* occurs directly from the division of the small phloem cells (pl. 18). The sections of the flax both in cross and long views show the regenerated parts arising from the epidermis cells. First the epidermis divides and then the innermost row of those cells and the stimulated cells of the region just beneath form the regenerated part, root or shoot (pl. 16).

Conclusions

Regeneration occurs in the (a) flax stem from the division of the epidermal cells; (b) *Bryophyllum* leaf at the notches from the division of the phloem cells of the veins; (c) sweet-potato, horseradish, and parsnip, from the division of the cambium cells.

Final Conclusions

Basing my conclusions on the instances cited in the literature and upon the laboratory experiments, I am convinced that regeneration occurs (1) from cambium cells when they are abundantly present and fully developed, as in the horseradish, sweet-potato, and parsnip, (2) from the young epidermal cells of seedlings before the central cylinder has a well-developed cambium, as in the flax seedlings, and (3) from the small and actively dividing cells of the phloem, as from the veins of the leaves of *Bryophyllum*.

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Explanation of Plate

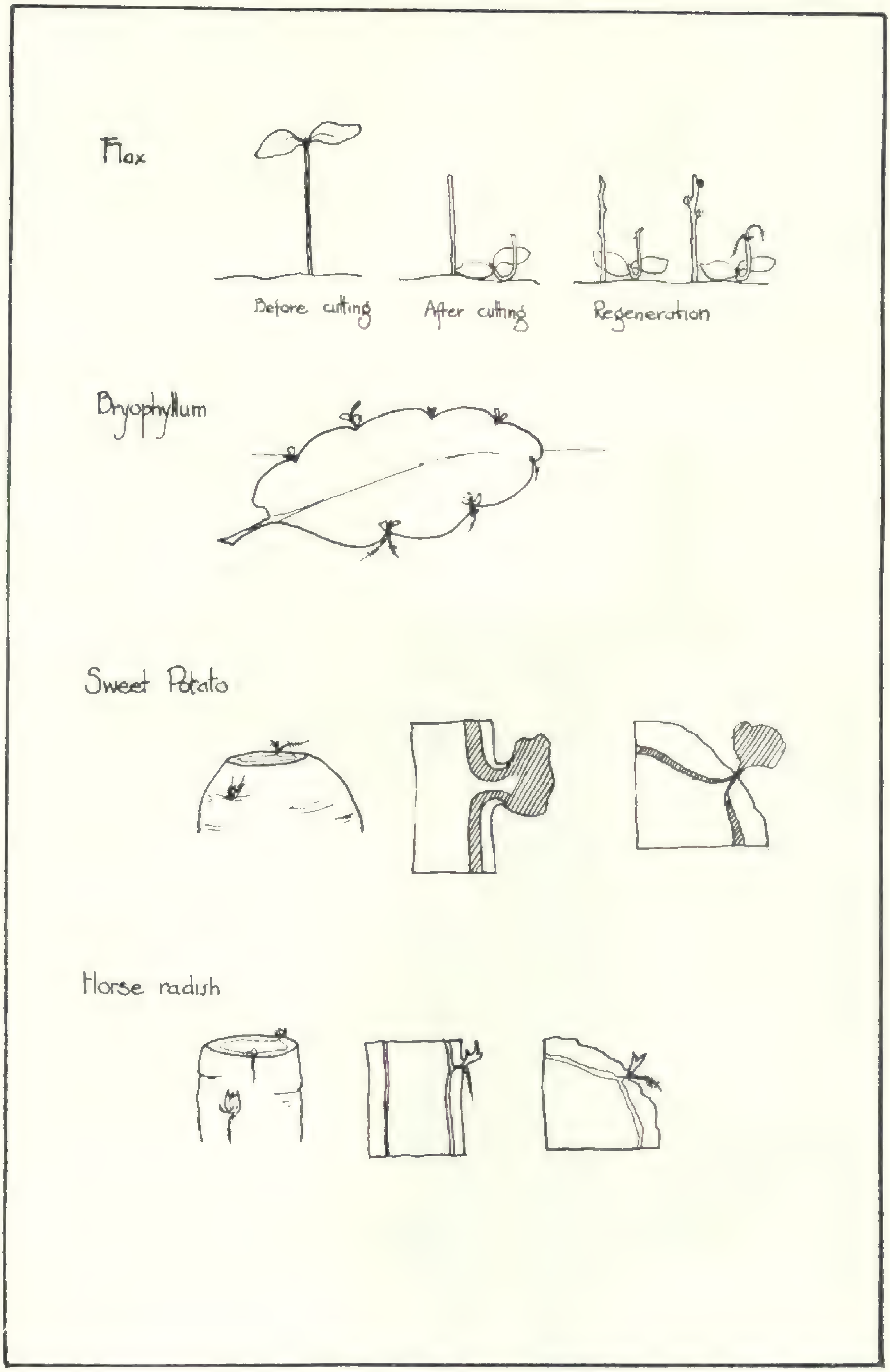
PLATE 15

The flax seedling is represented before and after cutting and shows regenerated roots and shoots.

The *Bryophyllum* leaf shows regenerated roots and shoots at the notches.

The sweet-potato has a regenerated shoot from the upper cut surface and one normally through the outer cortex.

The horseradish shows its similarity to the sweet-potato.

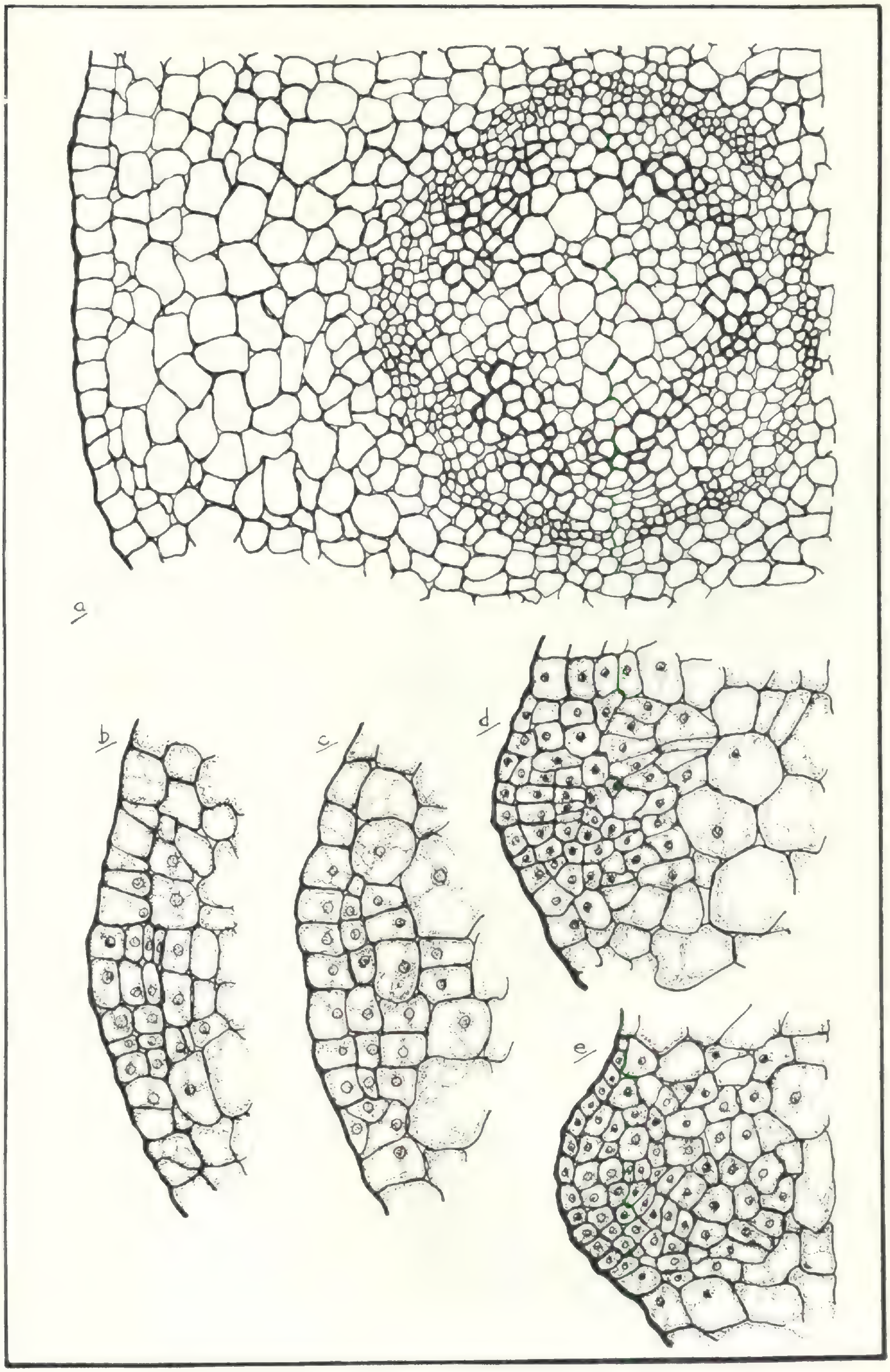


BEALS—REGENERATIVE PHENOMENA IN PLANTS

Explanation of Plate

PLATE 16

a. Normal cross-section through flax stem.
b. First divisions of epidermal cells.
c. Further divisions of inner rows formed in *b*.
d–e. Further development of regenerated bud.
(Camera-lucida drawings × 400.)



BEALS—REGENERATIVE PHENOMENA IN PLANTS

Explanation of Plate

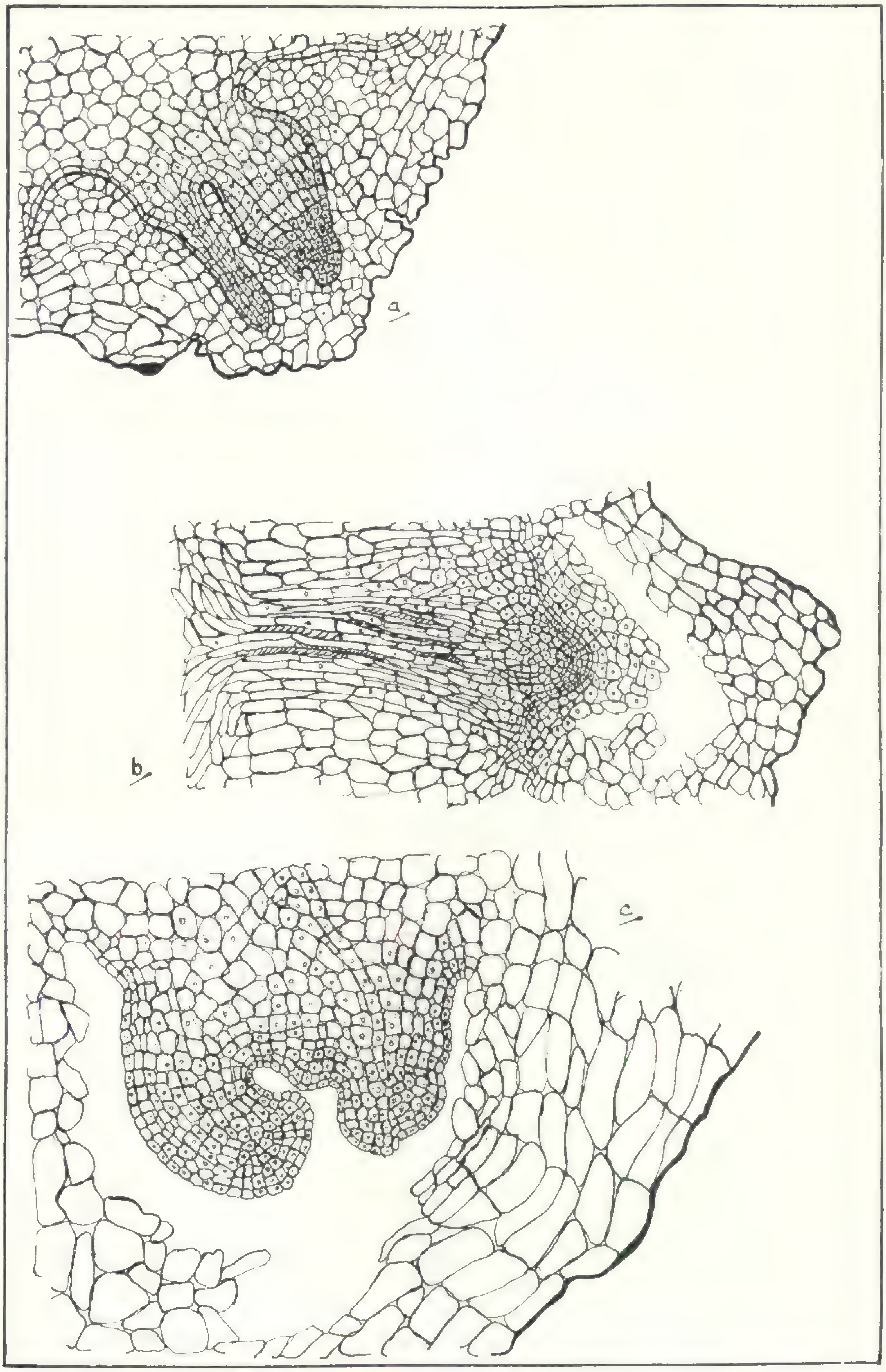
PLATE 17

a. Regenerated root of horseradish developing from some tissue other than the epidermis or cortex.

b. Regenerated part of horseradish showing direct connection with the cambium.

c. Regenerated bud of the sweet-potato developing in a manner similar to the root in the horseradish in *a*.

(Camera-lucida outlines × 143.)

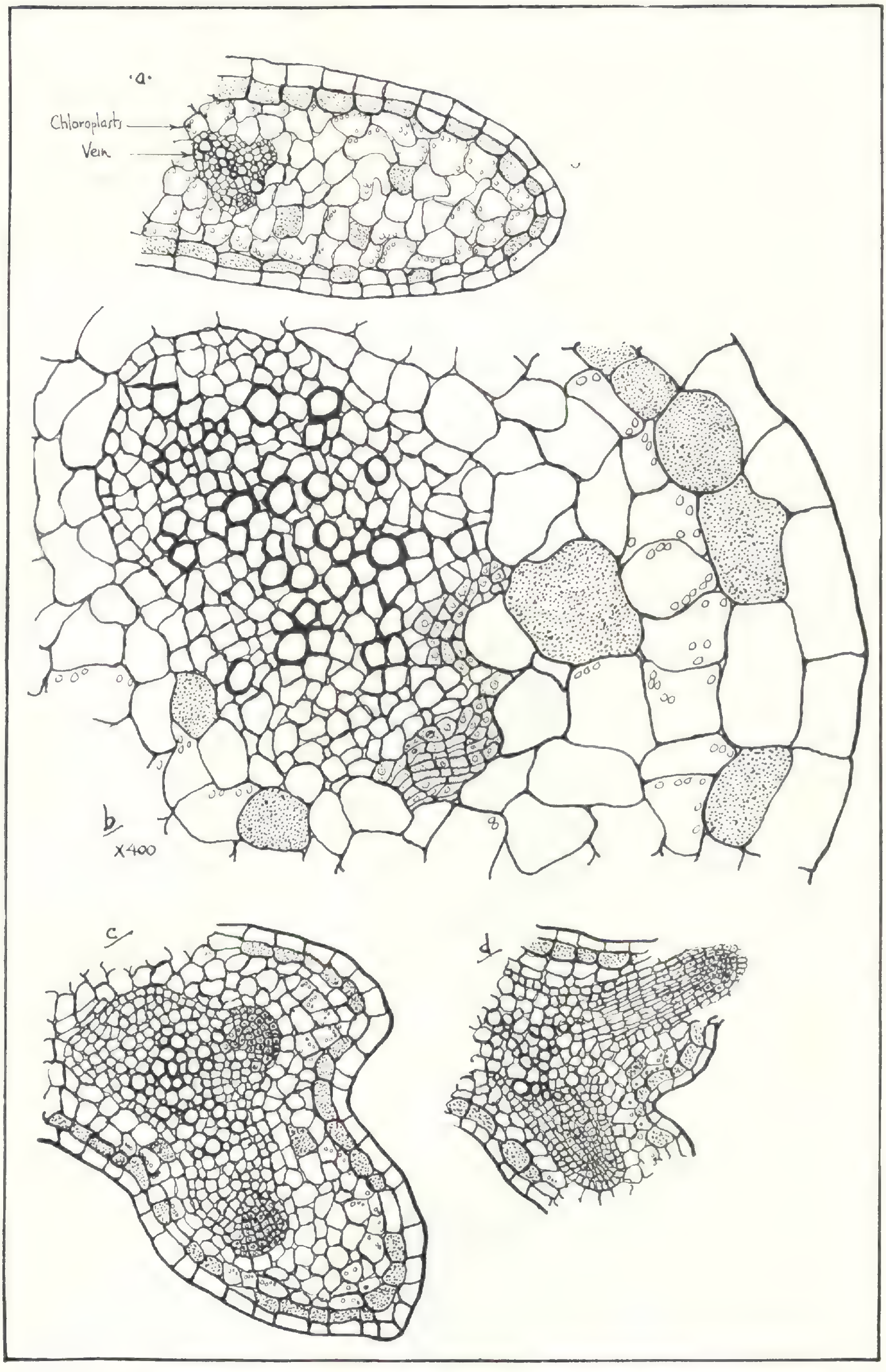


BEALS—REGENERATIVE PHENOMENA IN PLANTS

Explanation of Plate

PLATE 18

a. Cross-section of *Bryophyllum* leaf showing vein.
b. Camera-lucida drawing of vein.
c. Further division of small phloem cells of vein.
d. Regenerated root development from small cells of vein.



BEALS—REGENERATIVE PHENOMENA IN PLANTS

NOTES ON THE PHYSIOGRAPHY OF NORTH DAKOTA AND THE CONDITIONS IN CERTAIN OF ITS WATERS

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The following notes on the physiography of North Dakota and the conditions in certain of its waters are intended as an introduction to the paper by Dr. Moore and Dr. Carter on the plankton algae of these waters.

North Dakota may be divided into three main areas: (1) the Red River Valley, occupying the basin of glacial Lake Agassiz; (2) the drift prairie plain, a region covered with glacial till and boulders and of irregular surface, which slopes gradually upward from the valley to (3) the Missouri Plateau, an elevated table-land occupying the southwestern half of the state. The latter area is deeply cut by the valley of the Missouri River, a little to the east of which, and running nearly parallel with it, is an irregular line of low hills, constituting the Altamont Moraine, which marks the southwestern edge of the glacier which in the last glacial epoch covered the eastern half of the state, and whose drainage, in its retreat, formed Lakes Agassiz, Souris, and Saskatchewan. Southwest of the Missouri River is the greatly eroded region known as the "badlands," which constitutes the most picturesque feature of the state.

In the northern part of the area, on the Canadian boundary, is a group of low, irregular hills constituting the Turtle Mountains. These hills rise from 120 to 180 meters above the surrounding plain, and represent a portion of the Missouri Plateau, isolated therefrom by erosion, prior to the advent of the glaciers, and covered by the latter with till to a depth of from 30 to 60 meters. Among these hills are numerous spring-fed, fresh-water lakes, the largest of which, just east of Bottineau, has an area of about 6 square kilometers.

Between the Turtle Mountains and the Missouri Plateau lies the broad flat plain of the Mouse River occupying the basin of the glacial Lake Souris which extended northward into Canada.

The lakes considered in the present report are found in the drift prairie plain and Turtle Mountains. The drainage of the areas is very poor and numerous depressions among the hills form the basins of many of the lakes. While most of them are merely depressions in the land filled with water, there are several which are either expansions of an existent river near its head-waters, or which occupy portions of an old river channel. The largest of these are Devils Lake and Stump Lake, which were at one time parts of the much larger glacial Lake Minnewaukon, draining into the Sheyenne River.

Another group of these lakes is found in the course of the James River, north of Jamestown, and comprises Arrowhead, Jim, and one or two smaller lakes.

Spiritwood I and II and a number of neighboring lakes probably occupy an old river valley and the same is very evident in the case of Strawberry, Long, Crooked, and Turtle Lakes, north of Washburn.

Most of the lakes are shallow, having a depth of not more than 3–4.5 meters, while many are merely shallow pools which dry up in dry years, but may have a considerable extent in years of heavy rainfall. The deepest of any is Alkali Lake, II, which has a maximum depth of 27 meters. Their supply comes partly from springs, partly from run-off of rain or melting snow. In most cases this supply is inadequate to meet the demand and the lakes are gradually drying up. This is notably true of such highly alkaline bodies as Devils and Stump Lakes, but even in the case of spring-fed, freshwater lakes in the Turtle Mountains there has been a marked decrease in level in recent years. This drop may be due in part to the general lowering of the water table throughout the state in recent years, which in turn is probably due to continued opening of new artesian wells and their uncontrolled flow.

Most of the lakes under consideration have no outlet and this, coupled with their decrease in level in many instances, has led to the high concentration of salts present in so many of them.

Where the lakes have an outlet, either permanently or at times of high water only, as in the case of Arrowhead and Jim Lakes, already mentioned, the salts washed into the lakes by run-off from the drainage area are carried out by the outflow. In the case of lakes which are completely land-locked but yet are not markedly alkaline, it is probable that acids carried in by run-off from humus-covered areas, neutralize the alkalies in the water and largely precipitate them in the form of insoluble salts. The presence of organisms in the water, especially animals, may play a small part in reducing alkalinity through the production of carbon dioxide.

With reference to their chemical character it is not possible to make any definite classification of the lakes. They range all the way from those of a distinctly freshwater type, with low alkalinities, to exceedingly brackish waters with total carbonate alkalinities running up to 2000 ppm. and more.

But little attention has been paid to physical features, such as temperature, turbidity, and color, in most cases. Where the lakes are shallow there is little temperature difference between surface and bottom, and the summer temperature frequently runs up to 25° C. or more, especially near shore. In the deeper lakes several degrees difference may exist between surface and bottom. The determinations, however, in most of the lakes are too few to permit of any generalizations.

The larger plants comprise chiefly *Ruppia maritima*, which occurs abundantly in practically all the lakes of the region; *Potamogeton*, *Myriophyllum*, *Ceratophyllum* and *Chara*, all of which are common in fresh water; while *Juncus*, *Carex*, and *Scirpus* are abundant in the shallower parts of all but the more alkaline lakes.

The location of each lake is indicated on the accompanying map (pl. 20). In considering the various lakes, no definite classification can be made, either in the character of the water, or in the nature of shores and bottom. Several of the freshwater lakes, especially such as are merely expansions of a river, are very shallow, largely overgrown with rushes and sedges, and with muddy bottoms. Others, such as Spiritwood and Wood Lake, have, in part, rocky, steeply sloping shores with comparatively

free water close to shore, while in other parts the shores may be gently sloping, with sandy or muddy bottom, and largely overgrown with rushes, sedges, and pond-weeds of various sorts. The alkaline lakes in general lack the abundant growths of these plants, but their place is largely taken by *Ruppia* and the bottom is more apt to be covered by fine black ooze, the result of accumulation of large amounts of wind-borne dust and decaying plant and animal life. Both phyto- and zoo-plankton are generally abundant in all of the lakes, especially the former, which frequently forms a "water-bloom" on the surface.

The lakes may be grouped roughly into (1) freshwater, those with total alkalinities below 400 ppm., (2) intermediate, with alkalinities between 400 and 700 ppm., and (3) alkaline lakes with alkalinities above 700.

(1) Freshwater lakes. Here may be included those in the Turtle Mountains (Carpenter, Crow, Dion, Fish, Gravel, Gordon, Jarves, Metigosche, and Willow), and those in the James River Valley (Arrowhead and Jim Lakes), Court, Crooked, Ensign, near Frettem, Fort Totten, Juanita, Long Lake, near Ruso, Painted Woods, Red Willow, near Binford, Spiritwood I and II, Strawberry, South Twin (Lake Y) and Wood Lakes, besides a few others, which are mere ponds formed by the outflow of springs (Cut-off, Eastgate's, and Wheeler's Ponds I and II, near Stump Lake, and ponds H and I at the Odessa Narrows).

(2) Intermediate Lakes. Blue, Brush, Clear, Florence, Long Lake, near Binford, Sweetwater, South Free Peoples, Williams, and X.

(3) Alkaline lakes are those of the Devils-Stump Lake complex, including Main, East, Lamoreau, Stump, Mission, and Spring Lakes, and Lakes A, C, N[1], O[1] and P, besides Alkaline Lakes I and II, Buffalo, Coe, Etta, Four-Mile, North Free Peoples, Isabelle, Tokio, Twin, North and South Washington Lakes, Lake Z, and the ponds south and southwest of Brush Lake.

For most of the lakes only alkalinities have been determined. Of several, however, general analyses have been made. Three of

[1] These are temporary pools formed by rain and melting snow, occupying depressions in the floor of the old Devils Lake. Consequently they vary greatly from time to time.

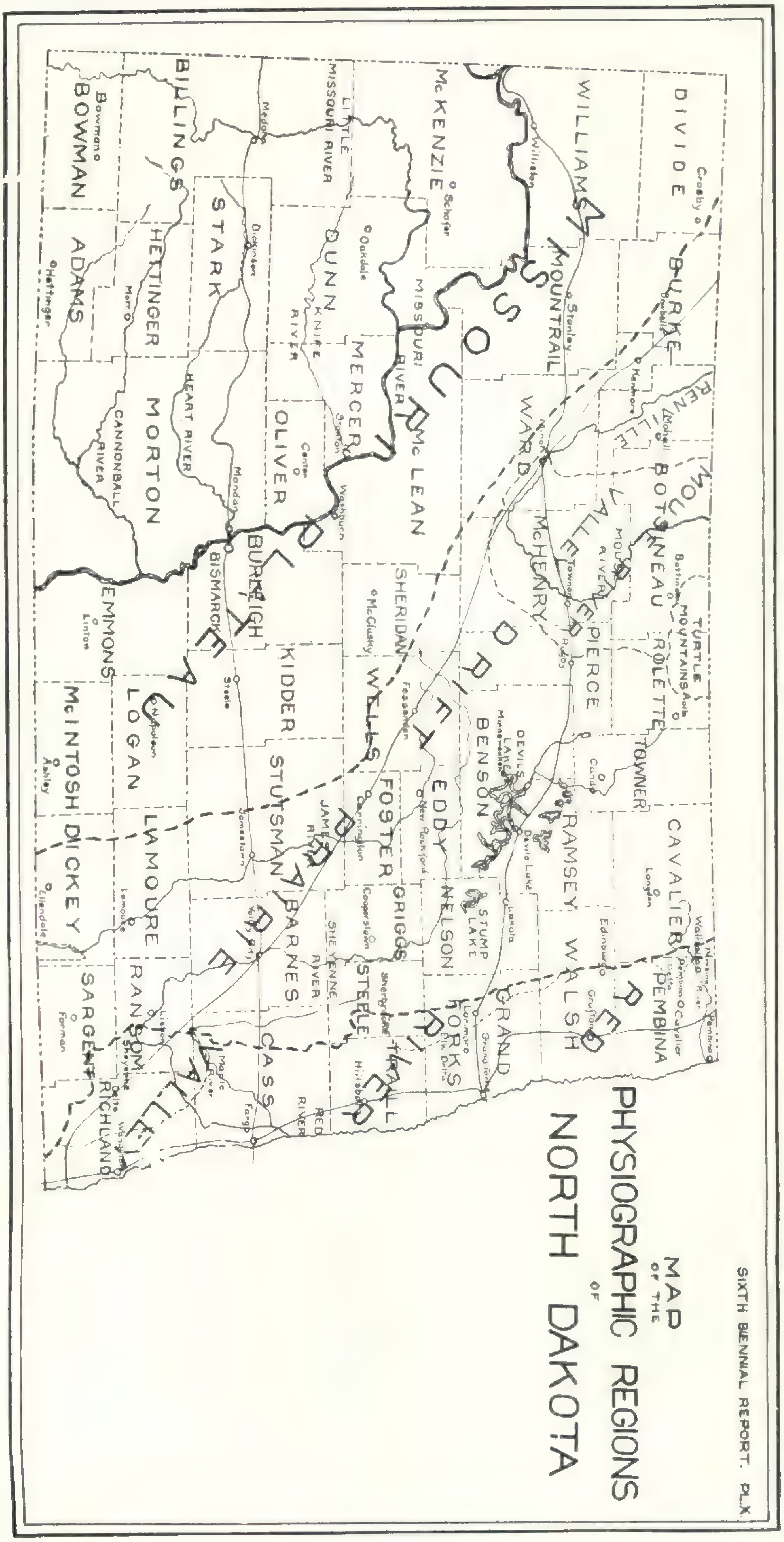
them, which are fairly representative of the three classes into which the lakes under discussion have been grouped, are given in the accompanying table.

Source	CO_3	HCO_3	Cl	SO_4	Mg	Ca	Na	K	SiO_2	Fe_2O_3 and Al_2O_3	Residue
Ft. Totten Lake (1919)	60	15	10	166	27	34	31	5	Trace	Trace	386
Sweetwater Lake (1911)	—	496.5	10.0	177.7	45.8	162.1	13.9	.1	17.0	2.0	694.0
Devils Lake (1919)	305	458	131 0	7187	585	70	2548	204	62	95	13462

Explanation of Plate

PLATE 19

Map of the physiographic regions of North Dakota (from the sixth biennial report of the North Dakota Geological Survey. Courtesy of Dr. A. G. Leonard, Director).

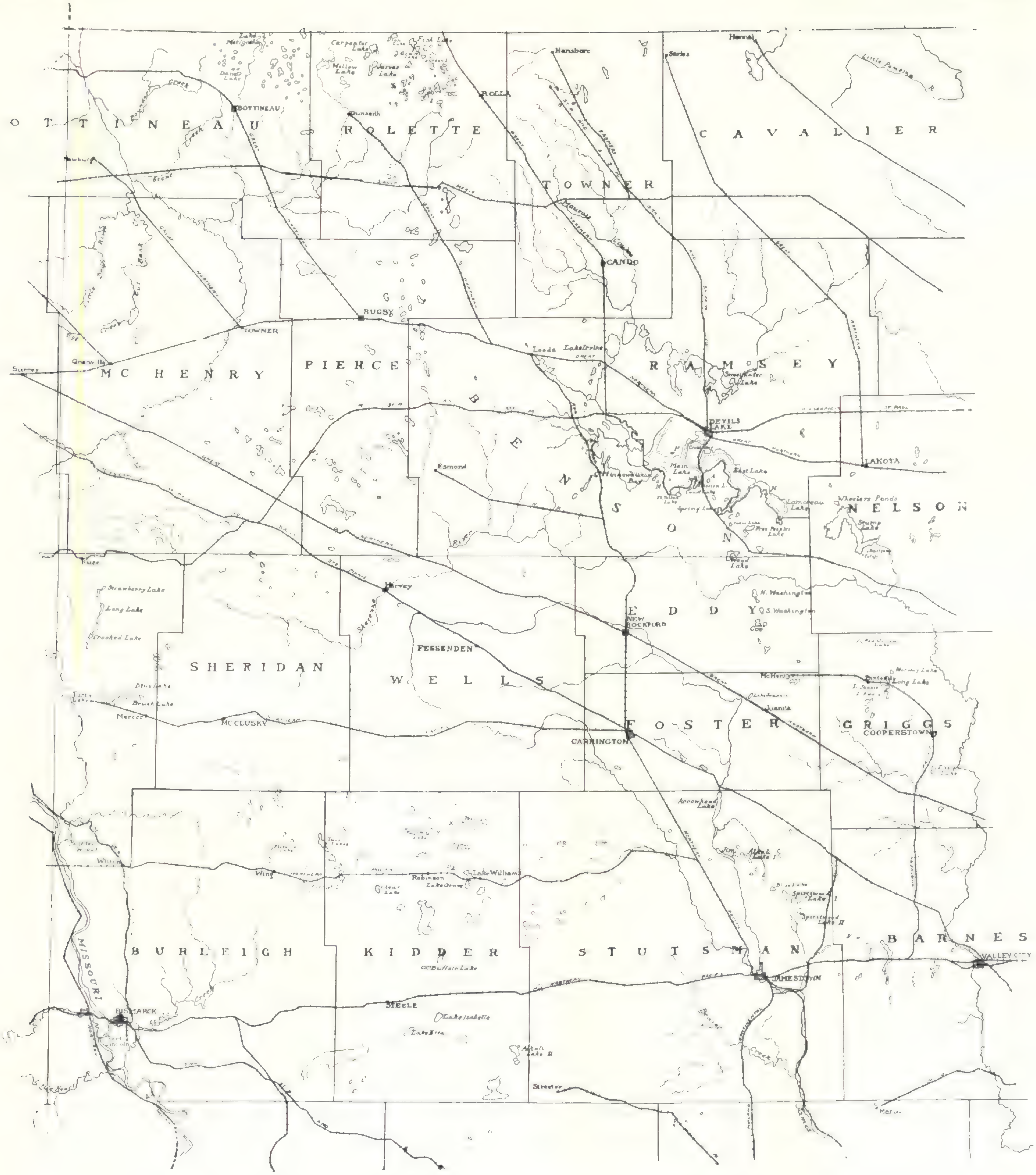


YOUNG—NOTES ON THE PHYSIOGRAPHY OF NORTH DAKOTA

Explanation of Plate

PLATE 20

Map of northeastern part of North Dakota (drawn by Wilfred C. Lowe).



YOUNG—NOTES ON THE PHYSIOGRAPHY OF NORTH DAKOTA

ALGAE FROM LAKES IN THE NORTHEASTERN PART OF NORTH DAKOTA

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In the Annals of the Missouri Botanical Garden, Vol. 4, Number 4, page 293, 1917, there was published a preliminary list of algae from Devils Lake, North Dakota. Since this list appeared there have been received from time to time, through Professor R. T. Young, Director of the State Biological Station, collections of algae, not only from Devils Lake proper, but also from many lakes in the surrounding regions. Because of the conditions under which many of these algae grow and the discovery of several forms which are extremely rare, or reported for the first time from this country, it seems desirable to publish from these more extensive samples a supplementary account of the algae from this particular section of North Dakota.

The list on p. 397 does not necessarily include all the species mentioned in the earlier list, and therefore in any consideration of the algal flora of this region it should be borne in mind that, since the preliminary list contains forms not appearing in the list published herewith, the two should be considered together in order to give the most comprehensive view of the algae from this region.

On pp. 395–396 the species from the area under consideration, both those observed in the present investigation and those contained in the earlier list, have been brought together with the object of contrasting the flora of the "freshwater" and "alkaline" lakes, according to the classification of the waters given above by Dr. Young. The number of "freshwater" and "alkaline" lakes

from which specimens have been received is about 30 in both groups. There were only 11 lakes of the "intermediate" group, and it was felt that the species observed in these would scarcely give a representative flora for comparison with those of the more numerous "alkaline" and "freshwater" lakes. The "intermediate" group has therefore been omitted from this comparative list.

From a consideration of the list it is apparent that as far as the *Myxophyceae* are concerned the "alkaline" lakes are considerably richer in species than the "freshwater" lakes. On the other hand, a definite "bloom" was rarely developed in strongly alkaline waters, whereas in several of the "freshwater" lakes there was frequently a copious "bloom" consisting of *Clathrocystis aeruginosa,* or sometimes of *Aphanizomenon flos-aquae,* one or other of these species being dominant and often the only representative of the *Myxophyceae* in the sample. In the alkaline lakes there was never such a pronounced development of a single species to form a "bloom," and the *Myxophyceae* were usually represented by various species of *Oscillatoria, Nodularia spumigena, Arthrospira Jenneri,* and *Spirulina* spp. The tiny species *Rhabdoderma sigmoidea,* described here for the first time, also occurred in some of the alkaline lakes as a dominant constituent.

With regard to the *Chlorophyceae,* the condition is reversed. Many of the species seem to be very intolerant of increasing concentration of salts, and are therefore eliminated from the flora of the strongly alkaline lakes. On the whole, the *Conjugatae* are very poorly represented, as pointed out by Moore ('17, *loc. cit.*, p. 302), although a few species are able to thrive under the unusual conditions. The genus *Oocystis* seems to maintain itself satisfactorily in the alkaline waters, as do also some species of *Scenedesmus, Pediastrum,* and *Dictyosphaerium* spp. Many of the *Protococcales* commonly found in freshwater plankton are not represented at all, however, in lakes of the "alkaline" group.

The "alkaline" lakes were conspicuous by reason of their paucity of species, the richest lakes of this type containing only 5–9 species, whilst some of the "freshwater" lakes had as many as 12–17 species. Odessa Pond, I, and Strawberry Lake were amongst the richest of the latter group, and Lake P and Coe Lake were the richest of the "alkaline" group.

TABLE I

SHOWING THE COMPARATIVE FLORAS OF THE "FRESHWATER" AND "ALKALINE" LAKES

Myxophyceae	Alk.	Freshw.
Chroococcus dispersus	*	*
var. minor		*
C. limneticus	*	*
C. turgidus	*	*
Gloeocapsa fenestralis		*
Chondrocystis Schauinslandii		*
Rhabdoderma sigmoidea	*	
f. minor	*	
Microcystis incerta	*	
M. pulvera		*
M. stagnalis	*	
Clathrocystis aeruginosa	*	*
var. major		*
C. holsatica		*
Gomphosphaeria aponina	*	*
var. cordiformis	*	*
Coelosphaerium Kuetzingianum	*	*
C. Naegelianum	*	*
Merismopedia glauca	*	
M. punctata	*	*
M. tenuissima	*	*
Holopedia irregularis		*
Tetrapedia Reinschiana		*
Oscillatoria amphibia	*	
O. brevis	*	*
O. chalybea	*	
O. chlorina	*	
O. geminata	*	
O. janthiphora	*	
O. limosa	*	
O. ornata	*	
O. tenuis	*	
Oscillatoria sp.	*	
Arthrospira Jenneri	*	
Spirulina major	*	
S. Nordstedtii	*	
S. subtillissima	*	
S. tenerrima	*	
Lyngbya birgei		*

Myxophyceae cont'd	Alk.	Freshw.
L. contorta	*	
L. Martensiana	*	
Nodularia spumigena	*	*
var. major	*	
Anabaena affinis		*
A. circinalis		*
A. flos-aquae	*	
var. gracilis	*	*
var. Treleasii		*
A. Lemmermanni		*
A. macrospora		*
A. spiroides	*	
Aphanizomenon flos-aquae	*	*
Plectonema tenue	*	
Tolypothrix lanata	*	
Calothrix Braunii	*	
C. fusca		*
C. parietina	*	
C. scytonemicola		*
Gloeotrichia echinulata		*
G. natans	*	
Rivularia coadunata		*
Asterocystis smaragdina	*	*
Total number of species and varieties	44	33

Chlorophyceae	Alk.	Freshw.
Conferva bombycina f. tenuis		*
Closterium Dianae	*	*
var. arcuatum	*	
Cl. eboracense	*	*
Cl. lanceolatum		*
Cl. Leibleinii	*	*
Cosmarium formosulum	*	*
var. Nathorstii		*
C. granatum	*	*
C. scopulorum	*	*
C. hians		*

TABLE I cont'd

Chlorophyceae cont'd	Alk.	Freshw.
C. impressulum		*
C. Meneghinii		*
C. pygmaeum		*
C. Regnellii		*
C. subcostatum	*	*
C. tenue		*
C. Turpinii		*
Arthrodesmus controversus		*
Staurastrum gracile		*
forma	*	*
St. paradoxum var. evolutum		*
Spirogyra lutetiana		*
Spirogyra sp.		*
Mougiotia calcarea		*
Mougiotia sp.		*
Pandorina morum	*	*
Eudorina elegans	*	
Volvox globator		*
V. mononae	*	
Botryococcus Braunii	*	*
Characium Hookeri	*	
Zoochlorella conductrix	*	
Oocystis crassa	*	
O. lacustris	*	*
O. pusilla	*	
O. solitaria	*	
Nephrocytium Naegelii	*	
Tetraëdron limneticum		*
T. quadricuspidatum		*
T. trigonum		*
var. gracile		*
Scenedesmus bijugatus	*	*
var. alternans		*
S. dimorphus		*
S. obliquus	*	
S. quadricauda	*	*
var. bicaudatus		*
var. quadrispina		*
var. Westii		*
Crucigenia quadrata	*	
C. tetrapedia		*
Selenastrum gracile		*

Chlorophyceae cont'd	Alk.	Freshw.
S. Westii		*
Ankistrodesmus falcatus		*
A. spiralis		*
Kirchneriella obesa		*
var. aperta		*
Coelastrum microporum	*	*
C. cambricum		*
Sorastrum spinulosum		*
Dictyosphaerium Ehrenbergianum	*	*
D. pulchellum	*	*
Actinastrum Hantzschii	*	*
Pediastrum angulosum	*	
P. boryanum	*	*
var. longicorne		*
P. duplex	*	*
var. clathratum		*
var. gracillimum		*
P. simplex		*
var. duodenarium		*
P. tetras		*
Ulothrix zonata	*	
Microspora Loefgrenii	*	
Enteromorpha intestinalis	*	*
E. prolifera	*	*
Enteromorpha sp.	*	*
Cylindrocapsa geminella		*
Oedogonium sp.	*	*
Bulbochaete sp.		*
Chaetophora elegans		*
Stigeoclonium nanum	*	
Stigeoclonium sp.		*
Herposteiron confervicola	*	*
Rhizoclonium crispum		*
R. hieroglyphicum	*	*
Cladophora Kuetzingianum	*	
Cladophora sp.	*	*
Chara elegans		*
Chara sp.		*
Total number of species and varieties	42	75

Flagellata

The plankton of North Twin Lake, near Wing, contained large quantities of an organism which should probably be referred to the genus *Euglena*, but which, because of poor preservation, could not be determined specifically.

Algae

Class MYXOPHYCEAE

Order COCCOGONEALES

Fam. Chroococcaceae

Genus chroococcus Näg.

Chroococcus dispersus (von Keissler) Lemm.

Cut-off Pond, Stump Lake; plankton of Lake P; plankton of pond, south of Brush Lake; North Washington Lake; plankton of Lake Isabelle; rare in the plankton of Tokio Lake; plankton of Lake Williams; abundant in the plankton of pond, southwest of Brush Lake.

Var. **minor** G. M. Smith in Wis. Geol. & Nat. Hist. Surv. Bull. 57: 28. *pl. 1, f. 3.* 1920.

Plankton of Crooked Lake.

C. limneticus Lemm.

Rare in the plankton of Carpenter Lake, near shore; more abundant in the deeper water of the same lake; plankton of Willow Lake; rare in the plankton of Brush Lake; abundant in the plankton of Florence Lake; plankton of Buffalo Lake; plankton of Long Lake, near Ruso; plankton of Lake X; plankton of South Free Peoples Lake; plankton of Carpenter Lake; North Free Peoples Lake.

C. turgidus (Kütz.) Näg.

Cut-off Pond, south end of Stump Lake; Alkaline Lake, II; North Free Peoples Lake; Lake P; Court Lake; slough below Wheeler's Pond; Main Lake.

Genus gloeocapsa Kütz.

Gloeocapsa fenestralis Kütz.

Also recorded by Snow (1902) from the plankton of Lake Erie. Eastgate's Pond.

Genus CHONDROCYSTIS Lemm.

Chondrocystis Schauinslandii Lemm. in Abh. Nat. Ver. Brem. 16^2: 353. 1899.

Associated with *Rivularia coadunata* encrusting a sunken board; only previously known from the Island of Laysan, Hawaiian group, Pacific.

Cut-off Pond, south end of Stump Lake.

Genus RHABDODERMA Schmidle & Lauterborn

Rhabdoderma sigmoidea sp. nov. Pl. 21, fig. 1.

Cellulis minutis, diametro circiter 1½–5- (plerumque 3-) plo longioribus; reniformibus, curvatis vel sigmoideis, rarissime ellipsoideis; apicibus rotundatis; solitariis, in familias haud consociatis; libere natantibus; contentu aerugineo homogeneo.

Long. 4–13 μ; crass. 2–3 μ.

Hab. In flora pelagica lacus, North Dakota.

Mission Bay; Mission Lake; East Lake.

Forma minor Pl. 21, fig. 2.

Cellulis minoribus et paullo gracillioribus.

Long. 5–10 μ; crass. 1.5 μ.

Lake P.

This minute alga occurred in great quantity and formed the principal constituent of the collections taken from the above-mentioned lakes. No trace of a gelatinous secretion leading to the formation of definite colonies could be detected, but it is possible that the long preservation to which the samples had been subjected (1–3 years in dilute formalin) might result in the disintegration of colonies of a delicate nature. The cells seemed to be floating quite independently of each other, only occurring rarely in short chains of two cells together, owing to slow separation after cell division. This new form differs markedly from *Rh. lineare*, the only other species of the genus, not only in its proportionate shorter and stouter cells, but also in its more distinctly curved and often sigmoid form. The forma *minor* is somewhat similar to the earlier described species but it is smaller and has the typical curved or sigmoid form of *Rh. sigmoidea*.

Genus MICROCYSTIS Kütz.

Microcystis incerta Lemm.

Lake P.

M. pulvera (Wood) Migula.

Plankton of Lake Metigosche; plankton of Jarves Lake; plankton of Strawberry Lake; plankton of Ensign Lake.

M. stagnalis Lemm. in Arkiv f. Bot. 2^{3}: 146. 1904 (*Polycystis stagnalis* Lemm. in Ber. d. deut. bot. Ges. **18**: 24. 1900; *Polycystis pallida* Lemm. in Bot. Centralbl. **76**: 154. 1898).

Previously known only from Europe.

Strawberry Lake.

Genus CLATHROCYSTIS Henfrey

Clathrocystis aeruginosa (Kütz.) Henfrey.

General and usually very abundant in the plankton of Wheeler's Pond, I, Stump Lake; Lake Metigosche; Jarves Lake; Strawberry Lake; Carpenter Lake, Fish Lake; Gravel Lake; Gordon Lake; Blue Lake; Juanita Lake; Long Lake, near Binford; Long Lake, near Ruso; Crooked Lake; Brush Lake; Florence Lake; Wood Lake; Spiritwood Lake, I; Jim Lake; North Twin Lake, near Wing; Lake Williams; Spiritwood Lake, II; South Washington Lake; Ensign Lake; Lake Y or South Twin Lake, near Robinson; Dion Lake.

Var. **major** (Wittr.) G. M. Smith.

Frequent with the typical form in the plankton of Ensign Lake; Carpenter Lake.

C. holsatica Lemm. in Plön. Ber. **10**: 150. 1903; Forti in De Toni, Syll. Alg. **5**: 95. 1907.

Plankton of Spiritwood Lake, II, near Jamestown.

Figures of the typical form of this species were not available. Comparison was made, however, with Lemmermann's figure of var. *minor* in Abh. Nat. Ver. Brem. **18**: *pl. 11*, *f. 1*. 1906, and the general appearance was very similar. In the size of the cells, however, and in the thick transparent gelatinous sheath of the colony, the North Dakota specimens agreed better with the description of the typical form. *C. holsatica* has only previously been known from Germany, and its variety from Siam.

Genus GOMPHOSPHAERIA Kütz.

Gomphosphaeria aponina Kütz.

Usually present in abundance in the plankton of Lake P; Strawberry Lake; Lake A; North Free Peoples Lake; Odessa, I; South Free Peoples Lake; Willow Lake; Wheeler's Pond; Lake Z, near Lake Williams; pond south of Brush Lake; North Washington Lake; Buffalo Lake; Coe Lake; rare in Tokio Lake; Creel Bay, Devils Lake; South Washington Lake; Lamoreau Lake; Lake C.

Var. **cordiformis** Wolle.

Cut-off Pond, south end of Stump Lake; Lake P; plankton of Lake Isabelle.

Genus COELOSPHAERIUM Näg.

Coelosphaerium Kuetzingianum Näg.

Strawberry Lake.

C. Naegelianum Unger.

Plankton of Gravel Lake; Gordon Lake; Juanita Lake; Crooked Lake; Spiritwood Lake, I; Coe Lake; Lake Isabelle; Lake Etta; Tokio Lake; Red Willow Lake; Carpenter Lake; North Free Peoples Lake.

Genus MERISMOPEDIA Meyen

Merismopedia glauca (Ehr.) Näg.

Slough below Wheeler's Pond; Crooked Lake; Lake Etta; Lake C.

M. punctata Meyen.

Cut-off Pond, Stump Lake; Lake A; Wheeler's Pond, II; Lake Williams; Red Willow Lake.

M. tenuissima Lemm.

Cut-off Pond, Stump Lake; Spring Lake; North Odessa, H; Wheeler's Pond; Lake P; Crooked Lake; Florence Lake; Lake Isabelle; Tokio Lake; Willow Lake; Lake C; Lake A.

Genus HOLOPEDIA Lagerh.

Holopedia irregularis (Lagerh.) Hansg. Prod. 2: 141. 1892 (= *Merismopedia irregularis* Lagerh. in Oefv. Kong. Sv. Vet. Akad. Förh. 1883[2]: 43. *pl. 1, f. 5, 6.* 1883).

Plankton of Brush Lake; Long Lake, near Ruso; Lake Williams; Ensign Lake; Red Willow Lake.

The dimensions of the present examples agreed very well with those given by Lagerheim, but the information given by him is not altogether complete. In the genus *Holopedia* the cells are usually ellipsoid in form, with their long axis perpendicular to the plane of the thallus. In the North Dakota specimens, however, the cells were nearly spherical, being approximately the same size in all three dimensions. Lagerheim states only the length and breadth of the cells, and does not say whether or not the cells were larger in the third dimension than the other two. The alga was very common in the plankton of Brush Lake, and here the colonies reached quite a considerable size. In the other localities it was not nearly so abundant, and usually occurred in small fragments. The species has previously been known only from Europe.

Genus TETRAPEDIA Reinsch

Tetrapedia Reinschiana Archer, Grevillea 1: 46. *pl. 3, f. 11–13.* 1872.

Red Willow Lake.

Order HORMOGONEAE

Fam. OSCILLATORIACEAE

Genus OSCILLATORIA Vauch.

Oscillatoria amphibia Ag.

Mission Bay, Devils Lake; East Lake.

Also recorded by Snow (1902) from the plankton of Lake Erie.

O. brevis (Kütz.) Gomont.

Wheeler's Pond, I; East Lake; mouth of Minnewaukon Bay, Devils Lake, below grade.

O. chalybea Mertens.

Mission Bay, Devils Lake; Mission Lake; Odessa, I; mouth of Minnewaukon Bay, Devils Lake, below grade.

O. janthiphora Fiorini-Mazzanti in Gom. Mon. Osc. p. 253. *pl. 7, f. 18, 20, 21.* 1893. Pl. 21, fig. 10.

Mission Bay, Devils Lake; Mission Lake; East Lake; mouth of Minnewaukon Bay, Devils Lake, below grade; inlet of Lake A.

This species seems to be very characteristic by reason of the curved and attenuated apex of its trichomes. It was usually present in great abundance and except in one locality the trichomes were always 7 μ in diameter. The specimens from the inlet of Lake A measured only 5 μ, but the character of the apex left no doubt as to their identity. It is believed that this is the first record of the species since its original discovery in Italy.

O. limosa Ag.

Stump Lake, spring.

? **O. ornata** Kütz. in Gom. Mon. Osc. p. 234, *pl. 6, f. 15.* 1893.

Amongst moss, near Stump Lake.

The identity of this alga was somewhat uncertain. In size and general appearance it was very similar to Kützing's species, but the trichomes were not at all attenuated at the apex, and although distinctly bent, were never spirally curved as figured by Gomont.

O. tenuis Ag.

Mission Lake; East Lake.

Genus ARTHROSPIRA Stizenb.

Arthrospira Jenneri (Kütz.) Stiz.

Mission Bay, Devils Lake; Mission Lake; East Lake.

Genus SPIRULINA Turp.

Spirulina major Kütz.

Plankton of East Lake; Spring Lake; mouth of Minnewaukon Bay, Devils Lake, below grade; Odessa, I; inlet of Lake A; Four Mile Lake; North Twin Lake, near Wing.

S. subtillissima Kütz.

Inlet of Lake A.

S. tenerrima Kütz.

Lake P.

Genus LYNGBYA Ag.

Lyngbya birgei G. M. Smith, Wis. Geol. & Nat. Hist. Surv. Bull. 57: 54. *pl. 7, f. 14, 15.* 1920.

Lake Metigosche; Crow Lake; Gravel Lake; Odessa, I; Juanita Lake; Wood Lake; Spiritwood Lake, I.

? **L. contorta** Lemm. in G. M. Smith, Wis. Geol. & Nat. Hist. Surv. Bull. 57: 53. *pl. 7, f. 12, 13.* 1920.

Plankton of Lake Isabelle.

This minute coiled species resembled greatly Smith's figures of *L. contorta* except that the filaments were much more slender, being less than 1 μ in diameter. *L. Rivularianum* Gomont is about the same size as the specimens from North Dakota, but in this species the trichomes are constricted at the joints and moreover it is not a plankton species. The diameter of *L. contorta* is given as 1.5–2 μ.

? **L. Martensiana** Menegh.

Lake O.

The alga was present in two samples, but in both cases was very fragmentary. The dimensions and thick sheath suggest that it should be referred to *L. Martensiana.*

Family NOSTOCHACEAE

Genus WOLLEA Born. et Flah.

Wollea saccata (Wolle) Born. et Flah. in Tilden, Minnesota Algae, p. 181. *pl. 8, f. 21, 22.* 1910.

Wheeler's Pond, I; slough below Wheeler's Pond.

This is the third locality for this interesting species, the alga having previously been recorded only from New Jersey and from Massachusetts.

Genus NODULARIA Mertens

Nodularia spumigena Mertens var. **genuina** Born. et Flah.

Stump Lake; East Lake; Creel Bay, Devils Lake; Lake A; Strawberry Lake; inlet of Lake A; North Washington Lake; Mission Bay, Devils Lake; Lamoreau Lake.

Var. **major** (Kütz.) Born. et Flah.

Lamoreau Lake.

Genus ANABAENA Bory

? **Anabaena affinis** Lemm.

Diam. trich. 5 μ; heterocysts spherical, 7 μ in diameter.

Slough below Wheeler's Pond.

The alga was present in abundance, but there were no spores so that its real identity is uncertain. The trichomes were quite straight, with a conical apical cell.

A. circinalis Rab.

Strawberry Lake.

A. flos-aquae (Lyng.) Bréb. var. **gracilis** Kleb. in Flora **80**: 268. *pl. 4, f. 23, 24.* 1895.

Frettem Lake; Carpenter Lake; Wheeler's Pond, I; Coe Lake.

This species was present in many of the collections although it was fruiting only in the one from Frettem Lake. The tangled trichomes were 4–6 μ in diameter, and closely resembled Klebahn's figure. The cells were more frequently spherical than elongated, but in all other characters the specimens agreed very well. One sample from Carpenter Lake contained a form of *Anabaena* with trichomes twisted into irregular spirals. Its trichomes were 5–6 μ in diameter, and somewhat immature spores were present, which were kidney-shaped but a little stouter than those of *A. flos-aquae* var. *gracilis* from Frettem Lake. The specimens were rather unsatisfactory, but the form is provisionally placed here.

Var. **Treleasii** Born. et Flah.

Diam. trich. 3.5–4 μ; spores 7 × 19 μ.

Strawberry Lake; Wheeler's Pond, I.

The coiled filaments of the specimens seemed to agree very well with this variety, except that the cells, although sometimes longer than broad, were usually approximately spherical. Spores were not observed in the material from Wheeler's Pond.

A. Lemmermanni Richter.

Jarves Lake; Lake Y or South Twin Lake, near Robinson; Carpenter Lake.

A. macrospora Klebahn var. **crassa** Klebahn in Flora **80**: 270. *pl. 4, f. 19, 20.* 1895. Pl. 21, figs. 17, 18.

Diam. trich. 10 μ; long. cell. 9–10 μ; heter. 12 μ; spor. (immat.) 25 × 15 μ.

Crooked Lake.

The trichomes were slightly larger than Klebahn's examples, but otherwise were very similar. The alga is striking by reason of its long rigid filaments, uniform except for the very rare

occurrence of heterocysts. Spores were also very rare, and being immature, were considerably smaller than the dimensions given by Klebahn, although in general form they were very similar to Klebahn's figure. West (Linn. Soc. Bot. Jour. **38**: 170. *pl. 9, f. 8.* 1907) records a similar rigid but sterile form of *Anabaena* from the plankton of Lake Tanganyika which differs only in the somewhat greater diameter of the trichomes, and the smaller proportionate size of the heterocysts.

A. spiroides Klebahn in Flora **80**: 268. *pl. 4, f. 11–13.* 1895. Pl. 21, figs. 11, 12.

Mission Bay, Devils Lake; Mission Lake.

Diam. trich. 8 μ; diam. spor. 11–13 μ.

The specimens were almost exactly similar to Klebahn's examples, the only difference being in the slightly smaller size of the spirals, which were about 30–40 μ in diameter, and the turns 30–40 μ apart, instead of 45–54 μ in the first case and 40–50 μ in the second as given by Klebahn. Spores were very abundant, and only very slightly longer than broad. The typical form has previously been known only from continental Europe, although var. *crassa* Lemm. has been recorded from the Wisconsin lakes and from Massachusetts.

Genus APHANIZOMENON Marr.

Aphanizomenon flos-aquae (L.) Ralfs.

General in most of the plankton samples, often pure and in abundance as a "water-bloom."

Wheeler's Pond, I; Crow Lake; Fish Lake; Coe Lake; Painted Woods Lake; Sweetwater Lake; Jim Lake.

Family RIVULARIACEAE

Genus CALOTHRIX Ag.

Calothrix fusca (Kütz.) Born. et Flah.

Crow Lake: growing in the gelatinous colonies of *Chaetophora elegans;* Devils Lake, amongst *Ruppia* near Station.

C. scytonemicola Tilden, Minn. Alg. p. 265. *pl. 17, f. 7.* 1910.

Dion Lake: growing in clusters on *Cladophora* sp.

Genus RIVULARIA Roth.

Rivularia coadunata (Sommerfelt) Foslie in Tilden, Minn. Alg. p. 291. *pl. 20, f. 16, 17.* 1910.

Cut-off Pond, Stump Lake: associated with *Chondrocystis Schauinslandii.*

R. nitida Ag.

Dion Lake.

Genus GLOEOTRICHIA Ag.

Gloeotrichia echinulata (Smith) Richter in G. M. Smith, Wis. Geol. & Nat. Hist. Surv. Bull. 57: 63. *pl. 11, f. 5, 6.* 1920.

Lake Metigosche; Strawberry Lake; Crow Lake; Carpenter Lake; Fish Lake.

Family GLAUCOPHYCEAE

Genus ASTEROCYSTIS Gobi[1]

Asterocystis smaragdina (Reinsch) Forti in De Toni, Syll. Alg. 5: 691. 1907 (*Callonema smaragdina* Reinsch, Contr. Alg. Fung. p. 41. *pl. 16.* 1875). Pl. 21, figs. 6–8.

Gordon Lake; Dion Lake; Lake Isabelle; Ensign Lake.

The figures of Reinsch illustrate an alga growing epiphytically on *Cladophora* with lax and almost dichotomous branching. The North Dakota specimens occurred in 4 different collections, 3 of which were plankton hauls. Only in two of the samples were the specimens at all frequent, and in one of these they were exclusively very young plants growing epiphytically. In the other three cases the specimens were distinctly free-floating. The young plants mentioned above consisted merely of short unbranched filaments, but in the better-developed and free-floating individuals, branching was usually very profuse (pl. 21, fig. 6). The lax branching figured by Reinsch was observed in only one individual, and in all others the branching was so intricate that the polarity of the plant could only be traced with great difficulty.

[1] The arrangement of De Toni (Vol. 5. 1907) has been followed in placing this problematical genus in the *Myxophyceae*. It is, of course, possible that its affinities are more with the *Bangiales*.

Intercalary cell division is apparently the rule, although growth at the apex is sometimes just as frequent. The cells seem to have a distinct membrane and are embedded in a thick gelatinous sheath of uniform width. There is a definite central body, probably a pyrenoid, which is distinctly visible even in material which has been preserved for some time in formalin (pl. 21, fig. 7).

Branches can apparently arise anywhere by the expulsion from the sheath of one of the intercalary cells, which then acquires a sheath of its own, and, by rapid and repeated division, may soon outstrip in length the filament which gave it origin (pl. 21, fig. 8). Although a branch thus formed often projects at right angles to the supporting branch, it may arise at almost any angle. Whilst branching by this kind of intercalary proliferation is most frequent, there is some evidence that branching at the apex of the filaments may also sometimes occur, although the exact method by which this is performed is not altogether clear. Sometimes a single cell with its individual sheath is seen projecting at right angles to the apical cell of a filament (pl. 21, fig. 8). How such a cell came to occupy this position is not apparent, but it is easy to see how it might, by repeated cell-division, give rise to one of those perpendicular branches so commonly encountered.

The alga has only previously been known from central Europe.

Class **HETEROKONTAE**

Order CONFERVALES

Family Confervaceae

Genus conferva Linn.

Conferva bombycina Ag. **forma tenuis** (Hazen) Collins.
Slough below Wheeler's Pond.

Class **CHLOROPHYCEAE**

Order CONJUGALES

Family Desmidiaceae

Genus closterium Nitzsch.

Closterium Dianae Ehrenb.
Outer margin 120–140° of arc; length 190–220 μ; breadth 18 μ.

Odessa, I; Lake P.

The specimens were rather smaller than usual, but were otherwise typical.

Var. **arcuatum** (Bréb.) Ehrenb.

Outer margin 150° of arc; length 150–164 μ; breadth 17–18 μ.

Lake Etta; Lake P.

Cl. eboracense Turn. (= *St. cucumis* Wolle, Desm. United States, *pl. 7, f. 17, 18.* 1892). Pl. 21, fig. 9.

Outer margin 100–110° of arc; length 240–250 μ; breadth 40–43.5 μ.

North Twin Lake, near Wing.

The specimens agreed very well with the figures and dimensions given by Wolle, although they are considerably larger than British examples according to West, Brit. Desm. 1: 140. *pl. 16, f. 7, 8.* A striking peculiarity is the frequent sigmoid form of the cells, nearly every cell being twisted so that the true curvature is very difficult to obtain. As far as could be ascertained the only species hitherto known which possesses sigmoid cells is *Cl. sigmoideum* Nordst. & Lagerh. (West, Brit. Desm. 1: 153. *pl. 19, f. 1–4*), but this species is very much more slender than the present specimens which, except for this peculiarity, are typical examples of *Cl. eboracense* Turn.

Cl. lanceolatum Kütz.

Outer margin 32–42° of arc; length 365–500 μ; breadth 50–62 μ.

Odessa, H; Odessa, I; Eastgate's Pond.

Cl. Leibleinii Kütz.

Outer margin 120–150° of arc; length 100–135 μ; breadth 15–18 μ.

Odessa, I; North Twin Lake, near Wing.

The specimens were not exactly typical, since there was only a slight and often scarcely perceptible median swelling instead of the pronounced one usually present. They seemed in many ways intermediate in form between *Cl. Leibleinii* and *Cl. Dianae,* but they were certainly too stout to be referred to any form of the latter.

Genus COSMARIUM Corda

Cosmarium formosulum Hoff.

Cut-off Pond, south end of Stump Lake; Lake P; Odessa, I; Eastgate's Pond.

Var. **Nathorstii** (Boldt) W. & G. S. West.

Cut-off Pond, south end of Stump Lake.

C. granatum Bréb.

Lake P; Odessa, I.

C. scopulorum Borge in Arkiv f. Bot. 18[10]: 12. *pl. 1, f. 14.* 1923.

Cut-off Pond, south end of Stump Lake; Lake P; Lake Isabelle.

C. hians Borge in Bot. Not. **1913**: 13. *pl. 1, f. 6.* 1913.

Length 19–21 μ; breadth 19–21 μ; isthmus 7–7.5 μ.

Odessa, I; Cut-off Pond, Stump Lake.

The specimens were slightly smaller than the original ones of Borge, but were otherwise exactly similar. The species has previously been recorded only from Sweden.

C. impressulum Elfv.

Eastgate's Pond.

C. Meneghinii Bréb.

Odessa, I.

C. pygmaeum Arch.

Cut-off Pond, Stump Lake; Eastgate's Pond.

A form with the end view elliptical and only one protuberance in the center of each lateral margin, the smaller ones on either side being absent.

C. Regnellii Wille.

Rare in Eastgate's Pond.

C. subcostatum Nordst. forma **minor** W. & G. S. West (= *C. calcareum* Johnson in Bull. Torr. Bot. Club **21**: *pl. 211, f. 13.* 1894).

Cut-off Pond, Stump Lake; Lake P; Odessa, I; Eastgate's Pond; Wheeler's Pond; Dion Lake; North Twin Lake, near Wing.

C. tenue Arch.

Cut-off Pond, Stump Lake.

C. Turpinii Bréb. var. **podolicum** Gutw.

Eastgate's Pond.

Johnson has recorded this form from Louisiana.

Genus ARTHRODESMUS Ehr.

Arthrodesmus controversus W. & G. S. West in Brit. Desm. **4:** *pl. 115, f. 12–14.* 1911.

Cut-off Pond, Stump Lake.

This minute desmid occurred in several samples and the regular splitting of the cells at the isthmus left no doubt as to its desmid character. Borge (in Arkiv f. Bot. **15:** 40. *pl. 3, f. 21.* 1918) has described a var. *brasiliense* from Brazil. The typical form has only previously been known from Europe.

Genus STAURASTRUM Meyen

Staurastrum gracile Ralfs.

Length, without processes, 17 μ, including processes, 17–18 μ; breadth, without processes, 8 μ, including processes, 33–36 μ; breadth of isthmus 4 μ.

Strawberry Lake; Carpenter Lake.

The specimens were very small, but exactly typical in form.

St. gracile Ralfs forma. Pl. 21, fig. 3.

Length, without processes, 37–42 μ, including processes, about 62 μ; breadth, without processes, 18–24 μ, including processes, 79–89 μ; breadth of isthmus 10–17 μ.

Strawberry Lake; Carpenter Lake; Red Willow Lake; North Twin Lake, near Wing.

This species is almost exactly identical with a certain form described by Dr. J. Lütkemüller as *St. Manfeldtii* Delp. var. *gracile* Lütk. from the plankton of a lake in Austria. This form was never published but appears under this name in a letter from Dr. Lütkemüller to the late Professor G. S. West, dated June, 1911. The dimensions of Lütkemüller's form are: length, without processes, 30 μ, including processes, 58 μ; breadth, without processes, 20 μ, including processes, 70 μ; breadth of isthmus 7.3 μ. The Austrian and the North Dakota forms are very similar. In both the processes are slightly divergent and the body of the semicell is cup-shaped. The chief difference lies in the somewhat deeper constriction and the stronger denticulations of the Austrian form, and the presence near the isthmus of a few granules beneath each process, not evident in the North Dakota specimens.

It is somewhat doubtful, however, whether this desmid should be placed as a form of *St. Manfeldtii*, which, with its short, stout processes, presents a somewhat different appearance to the alga under consideration, which has long graceful processes. By reason of this latter character it should probably be placed as a form of *St. gracile*.

St. paradoxum Meyen, var. **evolutum** West, Brit. Desm. **5**: 107. *pl. 145, f. 7–8.* 1923 (= *St. tetracerum* Ralfs var. *evolutum* West in Proc. Bot. Soc. Edinburgh **23**: 27. *pl. 2, f. 31.* 1905).

Forma **biradiata** Pl. 21, fig. 4.

Length, without processes, 7 μ, including processes, about 37 μ; breadth, without processes, 5 μ, including processes, 40 μ; breadth of isthmus 3.5 μ.

Strawberry Lake; Red Willow Lake.

This species bears some resemblance to *St. tetracerum* Ralfs and is somewhat similar to *St. tetracerum* var. *subexcavatum* Grönblad in Act. Soc. Fauna Flora Fenn. **49**: 62. *pl. 5, f. 28, 29.* 1921, which has processes considerably longer than the typical form. The North Dakota specimens, however, have still longer processes in comparison with the very minute body of the cell. The long slender processes suggest at once *St. subgracillimum* West in Trans. Linn. Soc. II. **5**: 263, *pl. 17, f. 3, 4.* 1896, but the form of the body is different, and the strong divergence of the processes precludes any relationship to that species. It is most likely that the alga should really be considered a biradiate form of *St. paradoxum* var. *evolutum*, which was described by the Wests from the plankton of lakes in the Shetland Islands. The cells were usually twisted at the isthmus, and there was a slight variation in the comparative length of the processes. The dimensions are only slightly smaller than those given by West.

Family Zygnemaceae

Genus zygnema Ag.

Sterile species of *Zygnema* were present in samples taken from Gordon Lake and Long Lake.

Genus SPIROGYRA Link

Spirogyra lutetiana Petit.

Odessa, I.

Sterile species of *Spirogyra* occurred in collections from Fort Totten, Cut-off Pond, Stump Lake, Odessa, I, slough below Wheeler's Ponds, and Painted Woods Lake, the latter, judging from the size of the filaments, being probably *Sp. maxima* (Hass.) Wittr.

Family MESOCARPACEAE

Genus MOUGIOTIA Ag.

Mougiotia calcarea (Cleve) Wittr.

Odessa, I.

Sterile forms of the genus were observed from Crooked Lake and Painted Woods Lake.

Order VOLVOCALES

Family VOLVOCACEAE

Genus PANDORINA Bory

Pandorina morum (Müll.) Bory.

Odessa, I; Minnewaukon Bay, below grade.

Genus EUDORINA Ehr.

Eudorina elegans Ehr.

Mouth of Minnewaukon Bay, Devils Lake, below grade; South Washington Lake (16-celled colonies).

Genus VOLVOX Linn.

Volvox globator Linn.

Wheeler's Pond, I.

? V. mononae G. M. Smith, Wis. Geol. & Nat. Hist. Surv. Bull. 57: 99. *pl. 18, f. 1.* 1920.

Mouth of Minnewaukon Bay, Devils Lake, below grade.

Probably this species but rather too badly preserved for a certain determination.

Family Tetrasporaceae

Genus botryococcus Kütz.

Botryococcus Braunii Kütz.

Main Wheeler's Pond, I; Long Lake, near Binford; North Washington Lake; Wood Lake; Lake Isabelle; Lake X; South Washington Lake.

Order PROTOCOCCALES

Family Scenedesmaceae

Genus oocystis Näg.

Oocystis crassa Wittr.

East Lake; Creel Bay, Devils Lake; Lamoreau Lake.

O. lacustris Chodat.

Willow Lake; Spring Lake; Lake C.

O. pusilla Hansg.

East Lake.

Genus tetraëdron Kütz.

Tetraëdron limneticum Borge.

Strawberry Lake.

T. quadricuspidatum (Reinsch) Hansg. Pl. 21, fig. 13.

Carpenter Lake.

A single triangular specimen, probably to be placed here, which possessed three stout spines and thickened convex sides.

T. trigonum (Näg.) Hansg.

Strawberry Lake.

Var. **gracile** (Reinsch) De Toni.

Red Willow Lake.

Genus scenedesmus Meyen

Scenedesmus bijugatus (Turp.) Lagerh.

Cut-off Pond. Stump Lake; Carpenter Lake; Willow Lake; Odessa, H; Lake P; Wood Lake.

Var. **alternans** (Reinsch) Borge.

Cut-off Pond, Stump Lake.

Var. **flexuosus** Lemm. Snow in Bull. U. S. Fish Comm. 22: 375. *pl. 1, f. 4.* 1902.

South Free Peoples Lake.

The coenobia were exactly similar to Snow's figure except that they consisted of 16 cells instead of 32. Both Miss Snow's specimens and those from North Dakota seem to differ from the original form of Lemmermann in the straight and uncoiled form of the coenobium. Lemmermann's figure (see G. M. Smith in Trans. Wis. Acad. **18**: *pl. 25, f. 16*. 1916), shows that his form was distinctly coiled in a spiral fashion, and G. M. Smith in his Monograph of *Scenedesmus* (*loc. cit.*, p. 446) has included this character in his description of the variety. The same writer (*loc. cit.*, p. 446) makes some reference to the spiral form of the coenobia in Miss Snow's work, but a careful examination of her paper has failed to show that she makes any statement concerning this character. Since her figures illustrate a perfectly straight colony, it has therefore been assumed that the specimens from Lake Erie differed from those of Lemmermann in exactly the same way as do those from North Dakota, in the straight form of the coenobia.

S. dimorphus (Turp.) Kütz.

Willow Lake; Dion Lake; Red Willow Lake.

S. obliquus (Turp.) Kütz.

Mouth of Minnewaukon Bay, Devils Lake, below grade.

S. quadricauda (Turp.) Bréb.

Cut-off Pond, Stump Lake; Willow Lake; Spiritwood I, Bay; Red Willow Lake; Carpenter Lake.

Var. **bicaudatus** Hansg. (= *Sc. longus* Meyen Smith, Wis. Acad. Sci. Trans. **18**: 469. 1915). Pl. 21, fig. 5.

Spiritwood I, Bay.

Var. **quadrispina** (Chodat) G. M. Smith.

Crow Lake.

Var. **Westii** G. M. Smith.

Crooked Lake.

Genus CRUCIGENIA Morren

Crucigenia quadrata Morren.

Rare in the plankton of East Lake.

C. tetrapedia (Kirchn.) W. & G. S. West.

Rare in the plankton of Red Willow Lake.

Genus SELENASTRUM Reinsch

Selenastrum gracile Reinsch.
Strawberry Lake.
S. Westii G. M. Smith, Wis. Geol. & Nat. Hist. Surv. Bull. 57: 133. *pl. 31, f. 10.* 1920.
Strawberry Lake.

Genus ANKISTRODESMUS Corda

Ankistrodesmus falcatus (Corda) Ralfs.
Dion Lake.
A. spiralis (Turn.) Lemm.
Strawberry Lake; Cut-off Pond, Stump Lake.

Genus KIRCHNERIELLA Schmidle

Kirchneriella obesa (West) Schmidle.
Red Willow Lake.
Var. **aperta** (Teiling) Brunnthaller.
Rare in Strawberry Lake.
K. lunaris (Kirchn.) Möbius, var. **irregularis** G. M. Smith, Wis. Geol. & Nat. Hist. Surv. Bull. 57: 142. *pl. 35, f. 1.* 1920.
Florence Lake.

Genus COELASTRUM Näg.

Coelastrum microporum Näg.
Cut-off Pond, Stump Lake; Spiritwood I, Bay; Coe Lake; Red Willow Lake.
C. cambricum Arch.
Strawberry Lake.

Genus SORASTRUM Kütz.

Sorastrum spinulosum Näg.
Cut-off Pond, Stump Lake.

Genus DICTYOSPHAERIUM Näg.

Dictyosphaerium Ehrenbergianum Näg.
Strawberry Lake; Creel Bay, Devils Lake.
D. pulchellum Wood.
Strawberry Lake; Juanita Lake; Clear Lake; Creel Bay, Devils Lake.

Genus ACTINASTRUM Lagerheim

Actinastrum Hantzschii Lag.

Wheeler's Pond, I; South Free Peoples Lake; North Washington Lake; Coe Lake; pond, southwest of Brush Lake; South Washington Lake; Creel Bay, Devils Lake.

Family HYDRODICTYACEAE

Genus PEDIASTRUM Meyen

Pediastrum boryanum (Turp.) Menegh.

Cut-off Pond, Stump Lake; Lake Metigosche; Jarves Lake; Strawberry Lake; Carpenter Lake; Lake O; East Lake; Lake A; Lamoreau Lake; Odessa, I; Eastgate's Pond; South Free Peoples Lake; Willow Lake; mouth of Minnewaukon Bay and Creel Bay, Devils Lake, below grade; Gordon Lake; Dion Lake; pond south of Brush Lake; Juanita Lake; Crooked Lake; Spiritwood, I, Bay; Brush Lake; Buffalo Lake; Florence Lake; Wood Lake; Long Lake, near Ruso; Clear Lake; Lake Isabelle; Lake X; Lake Etta; Tokio Lake; Lake Williams; Spiritwood Lake, II; pond southwest of Brush Lake; South Washington Lake; Ensign Lake; Lake Y or South Twin Lake, near Robinson; Lake C; Lake P; Mission Lake.

In many cases the two processes of each outer cell of the coenobium, although distinctly side by side, did not lie in the same plane, the one being in a much lower level than the other. They were not arranged, however, vertically, one above the other as in *P. Kawraiskyi* Schmidle.

Var. **longicorne** Racib.

Juanita Lake.

P. duplex Meyen.

Jarves Lake; Crow Lake; Carpenter Lake; Wheeler's Pond, I; Spiritwood Lake, I; Buffalo Lake; North Twin Lake, near Wing; Red Willow Lake; Long Lake, near Ruso; Coe Lake.

Var. **clathratum** (A. Br.) Lagerh.

Strawberry Lake; Wheeler's Pond, I; Juanita Lake; Brush Lake.

Var. **gracillimum** W. & G. S. West.

Strawberry Lake; Carpenter Lake; Gordon Lake; Florence Lake; Lake Williams.

P. simplex Meyen.
Strawberry Lake.
Var. **duodenarium** (Bail.) Rabenh.
Strawberry Lake.
P. tetras (Ehr.)Ralfs.
Cut-off Pond, Stump Lake; Odessa, I.

Order ULOTRICHALES

Family Ulvaceae

Genus enteromorpha Link.

? **Enteromorpha intestinalis** Link.
Mouth of Minnewaukon Bay, Devils Lake, below grade; Wheeler's Pond.
Probably this species but very fragmentary.
E. prolifera J. Ag.
Spring Lake; Wheeler's Pond, I; inlet of Lake A; Lake N; Stump Lake.
Enteromorpha sp. fragmentary.
Gravel Lake; Alkaline Lake, II; Odessa, H; Lake C; Devils Lake, near Station; Lake A; Lamoreau Lake.

Family Cylindrocapsaceae

Genus cylindrocapsa Reinsch

Cylindrocapsa geminella Wolle.
Odessa, I.
One short filament only found; fruit not present.

Family Oedogoniaceae

Genus oedogonium L.

Oedogonium sp. sterile occurred in samples from Stump Lake, Lake P, Lake O, Wheeler's Pond, I, Gordon Lake, Dion Lake, Strawberry Lake, North Twin Lake, near Wing, Devils Lake, near Station, Lake A.

Bulbochaete sp. with immature fruit in pond, east end of Spiritwood I, Bay.

Family Chaetophoraceae

Genus chaetophora Schrank

Chaetophora elegans (Roth) Ag.
Crow Lake.

Genus stigeoclonium Kütz.

Stigeoclonium sp. Pl. 21, figs. 14–16.
Eastgate's Pond.

The alga was a small epiphyte growing in great abundance on *Cladophora* sp., which was often entirely covered for lengths of 3 or 4 segments with its closely packed filaments (pl. 21, fig. 14). A number of epiphytic species of *Stigeoclonium* have previously been described, but the alga in question does not seem to agree with any one of them.

The thallus consists of two distinct parts—a basal portion of compact branching filaments, forming an almost parenchymatous stratum firmly applied to the wall of the *Cladophora*, and an upright system of usually short crowded branches. The upright branches are not usually more than 2 or 3 cells in length and rarely branch, but if branched may often give rise to long piliferous hairs (pl. 21, figs. 15, 16). These hairs show a cellular structure and are very abundant, being often developed in addition directly from the creeping portion of the thallus. Many of the cells in the erect filaments were very swollen, and their contents showed cleavage in preparation for the development of zoogonidia.

Of the previously known species of *Stigeoclonium*, the forms nearest to the North Dakota examples are the following:—*St. farctum* Berth. in Nova Acta Acad. Caes. Leop. **40**: 201. *pl. 16, f. 1–5.* 1878; *St. prostratum* Fritsch in Ann. S. Afr. Mus. **9**: 531. 1918; *Stigeoclonium* sp. Möbius in Hedwigia **26**: 238. *pl. 9, f. 3.* From *St. farctum* Berth. our alga differs in the shorter length of its erect filaments, which were rarely longer than 3 cells, whilst in Berthold's form they were 4–7. *St. prostratum* Fritsch differs in the somewhat laxer development of the basal portion of the thallus, and also in the fact that an upright system of branches is only present in certain regions, where the basal part of the

thallus is particularly lax. The basal creeping portion of the thallus is the more important in this species and the upright branches are very scanty in their development. *Stigeoclonium* sp. Möbius agrees with our species rather better than *St. farctum* in its relatively short erect filaments. Möbius, however, in describing his species states that in isolated cases these were observed to end in a hair. This seems to imply that hairs were of comparatively rare occurrence, whereas, on the other hand, our specimens were provided with numerous hairs. Taking all these things into account the alga under consideration seems to have greater affinities with *St. farctum* Berth., of which it is probably a form with poorer development of the erect part of the thallus.

Family Herposteiraceae

Genus herposteiron Näg.

Herposteiron confervicola Näg. (= *Aphanochaete repens* A. Br.).

Spiritwood I, Bay; North Twin Lake, near Wing.

Order SIPHONOCLADIALES

Family Cladophoraceae

Genus rhizoclonium Kütz.

Rhizoclonium crispum Kütz.

Wheeler's Pond, I.

Rh. hieroglyphicum (Ag.) Kütz.

Wheeler's Pond; Lake P; Lake N; Long Lake, near Binford; Lake Etta; Painted Woods Lake.

Genus cladophora Kütz.

Cladophora Kuetzingiana Grun.

Spring Lake; Lake O; Lamoreau Lake; Lake N.

Cladophora sp.

Cut-off Pond, Stump Lake; Fort Totten Lake; Gravel Lake; Lamoreau Lake; Odessa, I; Lake P; Lake A; Odessa, H; Dion Lake; Strawberry Lake; Alkaline Lake, II; North Twin Lake, near Wing; Painted Woods Lake; Lake O; Stump Lake; Lake P.

Order CHARALES

Family Characeae

Genus chara L.

Chara elegans (A. Br.) C. B. Robinson.
Cut-off Pond, Stump Lake.
Chara sp. fragmentary.
Spiritwood I, Bay.

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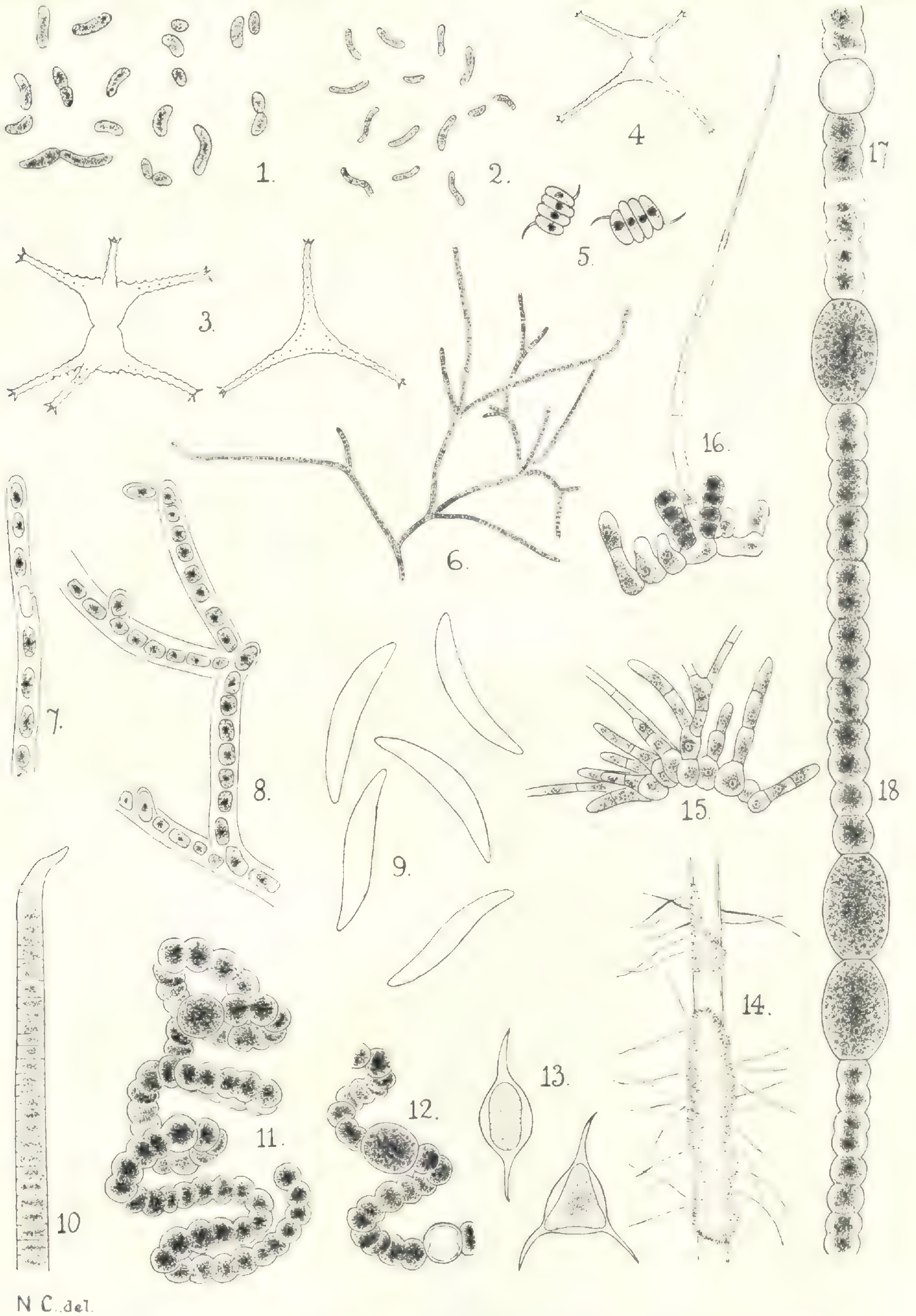
Description of Plate

PLATE 21

Fig. 1. *Rhabdoderma sigmoidea* sp. nov. × 570.
Fig. 2. *Rh. sigmoidea* forma *minor* × 570.
Fig. 3. *Staurastrum gracile* Ralfs forma × 335.
Fig. 4. *St. paradoxum* Meyen var. *evolutum* West × 570.
Fig. 5. *Scenedesmus quadricauda* (Turp.) Bréb. var. *bicaudatus* Hansg. × 570.
Figs. 6–8. *Asterocystis smaragdina* (Reinsch) Forti; fig. 6, × 90; figs. 7, 8, × 335.
Fig. 9. *Closterium eboracense* Turn. × 90.
Fig. 10. *Oscillatoria janthiphora* Fiorini-Mazzanti × 570.
Figs. 11, 12. *Anabaena spiroides* Klebahn × 570.
Fig. 13. *Tetraëdron quadricuspidatum* (Reinsch) Hansg. × 570.
Figs. 14–16. *Stigeoclonium* sp.; fig. 14, × 90; figs. 15, 16, × 570.
Figs. 17, 18. *Anabaena macrospora* Klebahn var. *crassa* Klebahn × 570.



MOORE AND CARTER—ALGAE FROM NORTH DAKOTA LAKES

GENERAL INDEX TO VOLUME X

New scientific names of plants and the final members of new combinations are printed in **bold face** type; synonyms and page numbers having reference to figures and plates, in *italic;* and previously published scientific names and all other matter, in ordinary type.

A

Acidity, relation of, to growth of citrus-destroying fungi, 245; to Diaporthe Sojae, 157
Actinastrum Hantzschii, 416
Aetheolaena involucrata, 83
Agaricus, 181
Alcoholic products, production of, by various fungi, 290
Aleurodiscus perideniae, 187
Algae from lakes in the northeastern part of North Dakota, 393
Alternaria Citri, 242, 281
Amino nitrogen, determination of, 330
Amoeba or protozoan theory of mosaic disease, 196
Anabaena affinis, 403; circinalis, 404; flos-aquae, 404, var. Treleasii, 404; Lemmermanni, 404; macrospora, 404, *422;* spiroides, 405, *422*
Ankistrodesmus falcatus, 415; spiralis, 415
Aphanizomenon flos-aquae, 405
Aphanochaete repens, 419
Apparatus and materials used in sugar determination, *72*
applanatus (Fomes), 184
arachnoideum (Corticium), 187
arcularius (Polyporus), 182
Armstrong, Joanne K., B. M. Duggar and. Indications respecting the nature of the infective particles in the mosaic disease of tobacco, 191
Arthrodesmus controversus, 410
Arthrospira Jenneri, 402
Asci of soybean fungus, *124,* containing ascospores, *122,* germinating, *124*
Aspergillus niger, 242; nitrogen metabolism in, 322
Asterocystis smaragdina, 406, *422*
auricula-Judae (Auricularia), 188
Auricularia auricula-Judae, 188; nigrescens, 188; tenuis, 188
australis (Fomes), 184

B

Bacillus atrosepticus, 5, 17, 43; caulivorus, 3; coli, 46; communis, 46; fluorescence liquefaciens, 3; melanogenes, 19; phytophthorus, 5, 17; solanisaprus, 18; vulgatus, 46
Bacterial theory of mosaic disease, 194
Beals, Cora Mautz. An histological study of regenerative phenomena in plants, 369
Blackleg, potato, 1; diagnosis of, 9; economic aspects of, 13
Botryococcus Braunii, 413
Bryophyllum, experiments in regeneration with, 373, *378, 384*
Bulbochaete sp., 417
Burt, E. A. Higher fungi of the Hawaiian Islands, 179

C

Cacalia arborea, 77; *dichroa,* 87; *glabrata,* 97; *involucrata,* 83; *patens,* 87; *pulchella,* 90; *teretifolia,* 96; *vaccinioides,* 97
Callonema smaragdina, 406
Calothrix fusca, 405; scytonemicola, 405
Camp, A. F. Citric acid as a source of carbon for certain citrus fruit-destroying fungi, 213
Campanella cucullata, 186
Carbohydrate utilization by strains of the blackleg bacillus and other microorganisms, 45
Carbon: analysis apparatus, 237, *238, 298;* citric acid as a source of, for certain fruit-destroying fungi, 213; total, determination of, 236
Carter, Nellie, George T. Moore and. Algae from lakes in the northeastern part of North Dakota, 393
cepaeforme (Lycoperdon), 189
Chaetophora elegans, 418

Chara elegans, 420
chioneus (Polyporus), 182
Chondrocystis Schauinslandii, 398
Chroococcus dispersus, 397, var. minor, 397; limneticus, 397; turgidus, 397
cinnamomea (Hymenochaete), 186
Citrate, growth of fungi on varying percentages of, 255
Citric acid, 213; apparatus, 222; as a source of carbon for certain citrus fruit-destroying fungi, 213; discussion of, 214; occurrence of, 226; physiological role of, 229; qualitative detection of, 215; quantitative determination of, 216
Cladophora Kuetzingiana, 419
Clathrocystis aeruginosa, 399, var. major, 399; holsatica, 399
Closterium Dianae, 407, var. arcuatum, 408; eboracense, 408, *422;* lanceolatum, 408
Coelastrum cambricum, 415; microporum, 415
Coelosphaerium Kuetzingianum, 400; Naegelianum, 400
coliforme (Myriostoma), 189
coliformis (Geaster), 189
commune (Schizophyllum), 181
Conferva bombycina f. tenuis, 407
Corium (Mycenastrum), 189
corrugata (Trametes), 185
Corticium arachnoideum, 187; **granulare,** 187
Cosmarium *calcareum*, 409; formosulum, 409, var. Nathorstii, 409; granatum, 409; hians, 409; impressulum, 409; Meneghinii, 409; pygmaeum, 409; Regnellii, 409; scopulorum, 409; subcostatum f. minor, 409; tenue, 409; Turpinii var. podolicum, 409
Crepidotus fulvotomentosus, 181; **rhizomorphus,** 181
Crucigenia quadrata, 414; tetrapedia, 414
cucullata (Campanella), 186
cucullata (Laschia), 186
cucullatus (Merulius), 186
Cylindrocapsa geminella, 417
Cyphella villosa, 186

D

Daedalea sanguinea, 185
Dextrose solution, 236, 323
Diaporthe Sojae, 128, *170–178*
Dictyosphaerium Ehrenbergianum, 415; pulchellum, 415
Diplodia natalensis, 242; nitrogen metabolism in, 322
dryophilus (Polyporus), 182
Duggar, B. M., and Joanne Karrer Armstrong. Indications respecting the nature of the infective particles in the mosaic disease of tobacco, 191
Duggar's solution, 323

E

elegans (Stereum), 187
Enteromorpha intestinalis, 417; prolifera, 417
Enzyme theory of mosaic disease, 194
Epithele **hydnoides,** 188
Eudorina elegans, 412
Exidia polytricha, 188

F

fasciatus (Fomes), 184
fasciculata (Poria), 184
fasciculatus (Fomes), 183
fibula (Polystictus), 184
flabellaris (Polyporus), 182
flabellatus (Pleurotus), 180
Flagellata, 397
Flax, experiments on regeneration with, 374, *378*, *380*
floccosus (Polystictus), 184
Fomes applanatus, 184; australis, 184; fasciatus, 184; **fasciculatus,** 183; Fullageri, 184; hawaiensis, 183; Korthalsii, 182; rimosus, 184; robustus, 184; *senex*, 182
Fullageri (Fomes), 184
fulvotomentosus (Crepidotus), 181
Fungi: certain citrus fruit-destroying, citric acid as a source of carbon for, 213; growth of, on varying percentages of citrate, 255; some aspects of nitrogen metabolism in, 299; studies in the physiology of the, XVI, 299

G

Ganoderma, 184
Geaster coliformis, 189
gemmatum (Lycoperdon), 189
gilvus (Polyporus), 182
glareosa (Psathyra), 181
Gloeocapsa fenestralis, 397
Gloeotrichia echinulata, 406
Glucose solution, 323
Gomphosphaeria aponina, 400, var. cordiformis, 400
granulare (Corticium), 187
Greenman, J. M. Studies of South American Senecios—I, 73
Grinding, effect of, on the infectivity of tobacco virus, 204

Gynoxis alternifolia, 76

H

hawaiensis (Fomes), 183
Hawaiian Islands, higher fungi of the, 179
Herposteiron confervicola, 419
Higher fungi of the Hawaiian Islands, 179
Hirneola nigra, 188
hirsutus (Polystictus), 184
Histological study of regenerative phenomena in plants, 369
Holopedia irregularis, 400
Horseradish, experiments on regeneration of roots of, 371, *378*, *382*
hydnoides (Epithele), 180
Hydnum, 186
Hydrogen-ion concentration of culture solutions, 325; change of media, 335; effect of, on germination of pycnospores of Diaporthe Sojae, 153; of culture solutions after growth of fungous mats, 258; of citric acid solution after fermentation by various fungi, 260; of dextrose solution after fermentation by various fungi, 259; reaction of, to growth of citrus fruit-destroying fungi, 245
Hymenochaete cinnamomea, 186; spreta, 186; tenuissima, 186

I

Indications respecting the nature of the infective particles in the mosaic disease of tobacco, 191

J

Jennison, H. M. Potato blackleg, with special reference to the etiological agent, 1

K

Kirchneriella lunaris var. irregularis, 415; obesa, 415, var. aperta, 415
Klotz, L. J. Studies in the physiology of the fungi, XVI, Some aspects of nitrogen metabolism in fungi, 299
Korthalsii (Fomes), 182

L

lactinea (Trametes), 185
Laschia cucullata, 186
latum (Stereum), 187
Lehman, S. G. Pod and stem blight of soybean, 111
Lemons, acid and sugar content of, 227
Lepiota xylophila, 180
Light, effect of, on production of pycnidia of various fungi, 147
lignosus (Polyporus), 182
Lycoperdon cepaeforme, 189; gemmatum, 189; Wrightii, 189
Lyngbya birgei, 402; contorta, 403; Martensiana, 403

M

marginata (Pholiota), 181
Media: employed in study of blackleg organism, 26; used in culture of soybean fungus, 153; used in nitrogen experiments, 323
Melilotus alba, 120
Merismopedia glauca, 400; *irregularis*, 400; punctata, 400; tenuissima, 400
Merulius cucullatus, 186
Microchaete corymbosa, 86; *glabrata*, 97; *pulchella*, 90; *trichopus*, 90; *vaccinioides*, 97
Micrococcus phytophthorus, 2
Microcystis incerta, 399; pulvera, 399; stagnalis, 399
microloma (Polystictus), 184
Moisture, effect of, on pycnidia formation, 152
Moore, George T., and Nellie Carter. Algae from lakes in the northeastern part of North Dakota, 393
Mosaic disease of tobacco, indications respecting the nature of the infective particles in the, 191
Mougiotia calcarea, 412
Mycenastrum Corium, 189
Myriostoma coliforme, 189

N

Naucoria triscopoda, 181
Nectria Ipomoeae, nitrogen fixation of, 334
nigra (*Hirneola*), 188
nigrescens (Auricularia), 188
Nitrites, determination of, 329
Nitrogen metabolism in fungi, some aspects of, 299
Nitrogen, total, determination of, 327
Nodularia spumigena, 403, var. genuina, 403, var. major, 403
North Dakota: algae from lakes in the northeastern part of, 393; map of the physiographic regions of, *390;* map of northeastern part of, *392;* notes on

the physiography of, and the conditions in certain of its waters, 383

O

Odontia Wrightii, 186
Oedogonium sp., 417
Oocystis crassa, 413; lacustris, 413; pusilla, 413
Oospora Citri-auranti, 244
Oranges, acid and sugar content of, 227
Oscillatoria amphibia, 401; brevis, 401; chalybea, 401; janthiphora, 401, *422*
ostreatus (Pleurotus), 180
Oxalic acid, determination of, 236

P

Pandorina morum, 412
Pediastrum boryanum, 416, var. longicorne, 416; duplex var. clathratum, 416, var. gracillimum, 416; simplex, 417, var. duodenarium, 417; tetras, 417
Penicillium digitatum, 241; italicum, 240; stoloniferum, 241
Peptone solutions, 323
perideniae (Aleurodiscus), 187
Peronospora infestans, 2
Persoonii (*Polystictus*), 185
Pholiota marginata, 181
Phoma Betae, 322; nitrogen metabolism in, 334
Phoma blight of soybean, 111
Phomopsis Citri, 244, 289
Physiography of North Dakota, notes on, and the conditions in certain of its waters, 385
pinsitus (Polystictus), 184
Pleurotus flabellatus, 180; ostreatus, 180
Pod and stem blight of soybean, 111, *170–178;* description of, 112; morphology of, 115
Polycystis pallida, 399; *stagnalis*, 399
Polyporus arcularius, 182; chioneus, 182; dryophilus, 182; flabellaris, 182; gilvus, 182; lignosus, 182; sulphureus, 182
Polystictus fibula, 184; floccosus, 184; hirsutus, 184; microloma, 184; Persoonii, 185; pinsitus, 184
polytricha (*Exidia*), 188
Poria **fasciculata,** 184; sp., 185
Potato blackleg, with special reference to the etiological agent, 1
Potato plants, affected with blackleg, showing characteristic symptoms, *70, 72*
Protozoan theory of mosaic disease, 197
Psacalium vaccinioides, 97; *glabratum*, 97
Psathyra, 181; glareosa, 181
Pycnospores of soybean fungus, *119;* germinating, *120*
Pycnosporophores of soybean fungus, *119*

R

Rainfall, relation of amount of, to prevalence of pod and stem blight of soybean, 145
Regenerative phenomena in plants, an histological study of, 369
Rhabdoderma **sigmoidea,** 398, *422*, f. **minor,** 398, *422*
Rhizoclonium crispum, 419; hieroglyphicum, 419
rhizomorphus (Crepidotus), 181
rimosus (Fomes), 184
Rivularia coadunata, 406
robustus (Fomes), 184

S

sanguinea (*Daedalea*), 185
Scenedesmus bijugatus, 413, var. alternans, 413, var. flexuosus, 413; dimorphus, 414; obliquus, 414; quadricauda, 414, var. bicaudatus, 414, *422*, var. quadrispina, 414, var. Westii, 414
Schizophyllum commune, 181
Sclerotinia Libertiana, 244, 287
Selenastrum gracile, 415; Westii, 415
Senecio **aberrans,** 73, *100;* abietinus, 75; adenophyllus, 75; adenotrichus, 75; **alternifolius,** 76; andicola, 76; apiculatus, 76; **arboreus,** 77; **aridus,** 77, *102; attenuatus* var. *microphyllus*, 80; auritus, 78; brachycodon, 78; **Buchtienii,** 78; Chinogeton f. macrocephalus, 80; **chaquiroensis** 79; clavifolius, 80; comosus, 80; culcitioides, 81; decompositus, 81; ericaefolius, 81; formosus, 82; *formosus*, 93, var. *subruncinatus*, 94; **genuflexus,** 82; involucratus, 83; iodopappus, 83; laciniatus, 84; latiflorus, 84; *ledifolius*, 76; **lophophilus,** 84, *104;* Magnusii, 85; medullosus, 85; Microchaete, 86; modestus, 86; nubigenus, 87; organensis, 87; otophorus, 87; patens, 87; pellucidinervis, 88; **Pennellii,** 88, *106*, var. **gachetensis,** 89; **pericaulis,** 89; *pimpinellifolius* var. *laciniata*, 84, var. *nubigena*, 87; pindilicensis, 90; pulchellus, 90; rudbeckiaefolius, 90; **sanctae-martae,** 91; **scortifolius,** 91; sinapoides, 92; Sprucei, 93; stigophlebius, 93; Stuebelii, 93; subdecurrens, 93; **subglomerosus** 93, *108;*

subruncinatus, 94; **sylvicolus**, 95, *110; Tabacon*, 82; teretifolius, 96; theaefolius, 97; tolimensis, 97; vaccinioides, 97, var. pruinosus, 97; *vernicosus* var. *major*, 76, var. *microphyllus*, 97; Weddellianus, 97; Xanthopappus, 98
Senecios, studies of South American—I, 73
senex (*Fomes*), 182
Soja max, 129
Solanum tuberosum, 1
Sorastrum spinulosum, 415
Soybean fungus, *170–178*
Soybean, pod and stem blight of, 111; cultural characters, 142; manner of infection, 139; overwintering and dissemination, 140; varietal susceptibility, 141
Sphaeropsis malorum, change in fungous mat, 340; nitrogen metabolism in fungi, 322
Spirogyra lutetiana, 412
Spirulina major, 402; subtillissima, 402; tenerrima, 402
spreta (Hymenochaete), 186
Staurastrum gracile, 410, forma, 410, *422;* paradoxum var. evolutum, 411, *422;* f. biradiata, 411, *422; tetracerum* f. *evolutum*, 411
Stereum elegans, 187; latum, 187
Stigeoclonium sp., 418, *422*
Stylospores of soybean fungus, *121*
Sugars, reducing, determination of, 48, 236, 325
sulphureus (Polyporus), 182
Sweet-potato, experiments on regeneration with, 371, *378*, *382*

T

tenuis (Auricularia), 189
tenuissima (Hymenochaete), 186
Tetraedron limneticum, 413; quadricuspidatum, 413, *422;* trigonum var. gracile, 413
Tetrapedia Reinschiana, 401
Tobacco, mosaic disease of, 191
Trametes corrugata, 185; lactinea, 185; sp., 185
triscopoda (Naucoria), 181

U

Ultrafiltration experiments in mosaic disease, 199
Ustulina vulgaris, 189

V

villosa (Cyphella), 186
Virus theory of mosaic disease, 195
Volvox globator, 412; mononae, 412
vulgaris (Ustulina), 189

W

Wollea saccata, 403
Wrightii (Lycoperdon), 189
Wrightii (Odontia), 186

X

xylophila (Lepiota), 180

Y

Young, R. T. Notes on the physiography of North Dakota and the conditions in certain of its waters, 385

Z

Zygnema, 411